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We have assessed the ability of *Wnt*-1, *Wnt*-2, *Wnt*-3, *Wnt*-3A, *Wnt*-4, *Wnt*-5A, *Wnt*-5B, *Wnt*-6, *Wnt*-7A, and *Wnt*-7B to transform mammary epithelial cells and found that Wnt-1, Wnt-2, Wnt-3 and Wnt-3A proteins transform mammary epithelial cells; Wnt-7A and Wnt-7B proteins partially transform; and Wnt-4, Wnt-5A, Wnt-5B, and Wnt-6 proteins does not affect mammary epithelial cells. Transformation correlated with Wnt-mediated increases in the cytosolic pool of β-catenin. By generating chimeric Wnt proteins and Wnt-1 deletions we have defined regions of Wnt-1 that are critical for transformation potential, signal transduction and frzb association. We have demonstrated that a secreted protein, frzb, blocks Wnt signaling. Wnt-1 acts as a mitogen in cultured Rat-1 fibroblasts, allowing growth of cells in serum-free medium. Using the TAC-2 mammary epithelial cell line, we found that Wnt-1 induces branching morphogenesis of mammary epithelial cells and can act as a morphogen in this capacity.

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WNT PROTEINS IN MAMMARY EPITHELIAL TRANSFORMATION
Final Report-1998

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INTRODUCTION

I. NATURE OF THE PROBLEM

There is strong evidence that Wnt proteins function as peptide growth factors that regulate the mammary gland growth cycle. Some of these proteins have already been shown to contribute to experimental mammary gland tumorigenesis in the mouse. Several groups are currently assessing whether *Wnt* genes play a role in the pathology of human mammary tumors, as might be predicted. Despite this evidence, work focused on the role of *Wnt* genes in breast cancer is in its infancy. The proposed studies are directly aimed at testing the hypothesis that *Wnt* genes encode regulators of normal and neoplastic mammary gland development.

The Wnt-1 protein is recognized as a mediator of cell-cell signaling events that can contribute to mammary tumorigenesis in the mouse. Despite accumulating evidence that Wnt-1 proteins act as growth factors, in the past it has been extremely difficult to purify Wnt-1 proteins in a soluble, cell-free form. For this reason, very little is known about Wnt specific cell surface receptors, which are proposed to be responsible for receiving signals from extracellular Wnt proteins. There is a pressing need to produce soluble, active Wnt ligands in order to understand the nature and regulation of Wnt-mediated growth control. In this proposal we will evaluate the hypothesis that *Wnt* genes encode a family of proteins that act as secreted growth factors that affect mammary epithelial cell physiology by interacting with cell surface receptors. It is expected that several of the Wnt proteins will demonstrably affect the growth properties of mammary epithelial cells, that these proteins act as secreted factors, and that they carry out their functions by stimulating specific cell-surface receptors on mammary epithelial cells.

II. BACKGROUND

The development of the mammary gland is a poorly understood process that consists of cycles of growth, morphogenesis, differentiation, and involution under the control of a variety of hormones and growth factors. Peptide growth factors have been implicated as effectors of mammary gland development (reviewed in [1]). Deregulation of growth factor-stimulated signaling pathways can contribute to the pathobiology of breast cancer [2-4]. Our current knowledge of growth factor involvement in mammary tumorigenesis has focused on the activation of tyrosine kinase signaling cascades. The *Wnt* gene family encodes growth factors that were originally identified by their ability to induce mammary gland tumors in mouse model systems. Wnts are involved in cell growth and cell fate determination during embryogenesis, organogenesis, and oncogenesis. Wnt proteins utilize a novel signal transduction pathway that may involve the frizzled, dishevelled, glycogen synthase kinase-3, catenins, and the tumor suppressor APC. Aberrant Wnt signaling is found in human colon cancers and melanomas. We are interested in the role of the Wnt signaling cascade in mammary tumorigenesis.

Wnt family genes

The first *Wnt* genes to be cloned were identified based on their oncogenic effects in the mouse mammary gland. The *Wnt*-1 gene (originally *int*-1[5])was identified as a frequent target for insertional activation by mouse mammary tumor virus (MMTV) proviral DNA in MMTV-induced mammary gland tumors[6,7]. Inappropriate expression of the *Wnt*-1 gene leads to mammary gland tumorigenesis[8,9]. *Wnt*-3, was also originally identified as a transcriptionally activated oncogene in MMTV-induced mammary tumors[10]. Most murine *Wnt* genes were isolated by cloning genes homologous to *Wnt*-1 [11, 12, 13] and encode cysteine-rich, secretory glycoproteins ranging from 350-380 amino acids. A comparison of the predicted amino acid sequences among murine *Wnt* gene family members reveal over 100 conserved residues fairly evenly distributed across the entire sequence and striking conservation of roughly 23 cysteines in nearly parallel positions. Different Wnt proteins are generally 40-60% identical at the amino acid level.

The normal functions of *Wnt* genes have been analyzed in organisms tractable to genetic or biochemical analysis of early development. The *Wnt*-1 orthologue in *Drosophila* is the segment polarity

gene *wingless* [11,12]. Genetic and biochemical analyses suggest that the wg protein functions as a local-acting, secreted factor that triggers a cascade of molecular events leading to the specification of segment polarity in the *Drosophila* embryo(reviewed in [13]). In the frog, *Xenopus laevis,* several different *Wnt* genes have been shown to contribute to the experimental induction of dorsal mesoderm tissue and subsequent establishment of the body axis [14-17]. Current models of early embryonic patterning events in the frog invoke one or several Wnt proteins as determinants of dorsal-axial position[17,18].

The murine *Wnt* genes cloned to date are expressed in spatially restricted patterns during gastrulation, neurulation, or early organogenesis. On the basis of the analysis of *Wnt*-1 gene deficiencies, the normal function of the murine *Wnt*-1 gene is in proper development of the cerebellum and midbrain [19,20]. Wnt family proteins are also implicated in limb development[21,22], kidney development[23,24], and uterine development[25]. In collaborative work with Dr. Andrew Lassar (Harvard University), we have also demonstrated that Wnt proteins, in combination with Sonic hedgehog (Shh), can induce myogenesis in somitic tissue *in vitro* [26]. This work indicates that myotome formation *in vivo* is directed by the combinatorial activity of Shh secreted by the floor plate and notochord and Wnt ligands secreted by the dorsal neural tube.

Wnt proteins and their mechanism of action

Wnt-1 encodes a secreted polypeptide characterized by a hydrophobic signal peptide, N-linked glycosylation sites, cysteine residues, and the absence of an identifiable membrane anchor domain [27]. Work on the biochemical properties of Wnt proteins has been carried out with cells programmed to express exogenous *Wnt* cDNAs. In such cells, Wnt-1 behaves as a secretory glycoprotein, undergoing entry into the endoplasmic reticulum (ER), leader cleavage, and asparagine(N)-linked glycosylation at several sites [28,29]. Despite entry into the ER, Wnt-1 proteins are very poorly secreted. Intracellular Wnt-1 is predominantly bound to BiP; a chaperonin-like protein found in the ER [30]. The appearance of extracellular Wnt-1 proteins is enhanced by addition of heparin sulfate [31] or suramin [32] to the media. Wnt-1 proteins thus are not freely diffusible once outside of cells. This lack of diffusibility is thought to be a result of tight association with either the cell surface [32] or the extracellular matrix [31]. Wnt proteins can act in a paracrine fashion as cell transformation assays have been used to define paracrine effects of Wnt-1[33,34]. Analysis of wg protein function, the *Drosophila* homologue of *Wnt*-1, suggests that it acts in a paracrine fashion [35]. These observations have led to the model that Wnt proteins are local-acting factors that function to signal cells adjacent or near the site of Wnt production but do not affect cells distant from the site of production. In fact, Wnt-1 proteins tethered to the cell surface by addition of a transmembrane tail still exhibit autocrine and paracrine transforming activities[36]. Wnt-specific activity can be detected in the medium of mammary epithelial cells programmed to express *Wnt*-1 cDNA ([37]and J. Kitajewski, unpublished observations). Biological activity of soluble wingless protein from cultured *Drosophila* imaginal disc cells has also been reported[38] . These studies suggest that Wnt proteins can act as diffusible secreted factors; however, the levels of soluble Wnt proteins are low. Despite such progress the purification of Wnt proteins has not yet been accomplished.

Wnt signal transduction events

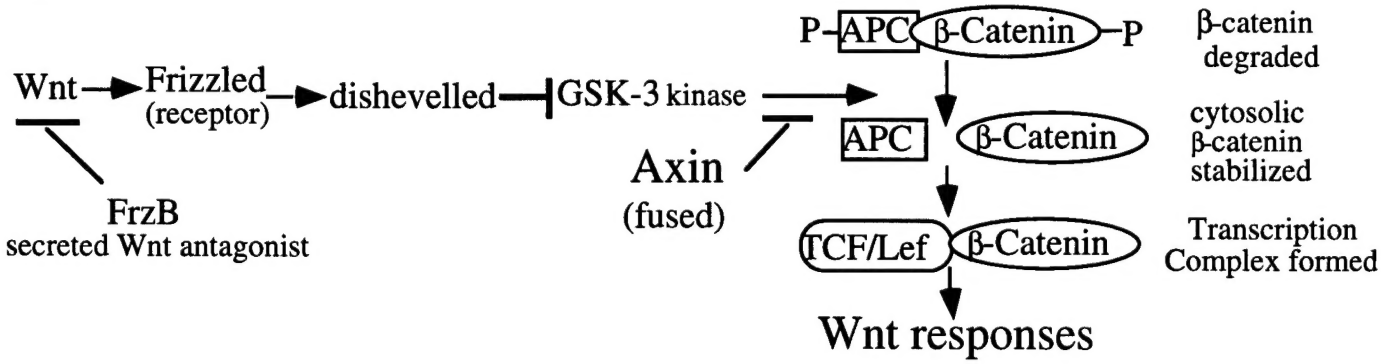
The nature of the signaling events triggered by Wnt proteins is now becoming apparent [39]. Genetic and biochemical analysis of the *wg* signal transduction pathway in *Drosophila* embryos and cultured insect cells suggest a cascade of events distinct from any previously described signal transduction pathway [13,40,41]. A model for the *wg*-mediated signaling pathway (schematized in Figure 1) has been proposed to involve the frizzled cell surface receptor [42]. Frizzled proteins contain a conserved amino-terminal cysteine-rich domain (the CRD domain) and seven putative transmembrane segments. Within the target cell, the cytosolic dishevelled protein is the first known component in *wg*-mediated signaling, however, its function is unclear. Dishevelled (dsh) may undergo hyperphosphorylation in response to wg protein (Roel Nusse, personal communication). Downstream of dsh is a protein kinase, zeste-white 3 (zw-3), whose activity must be suppressed to transmit wg signals. Suppression of the zw-3 kinase leads directly, or indirectly, to dephosphorylation and increased stability of the armadillo protein[43]. Increased cytosolic armadillo is then thought to form a complex with members of the Tcf/LEF-1 family of transcription factor which then move to the nucleus to regulate expression of wg target genes [44]. The *Drosophila* homologue of RhoA p21 GTPase has been genetically tied to frizzled function but it is not known how RhoA may function in Wnt signaling [45].

The components of the *Drosophila* wg signaling pathway have been conserved in vertebrates. Eight frizzled genes [46] and three *Dsh* genes [47] have been identified in the mouse or rat. *Drosophila zeste-white 3* (*zw-3*) encodes a serine/threonine kinase that is the homologue of mammalian Glycogen Synthase Kinase-3β [48]. Amadillo is the orthologue of vertebrate cadherin-associated molecules known as β-catenin and plakoglobin (γ-catenin) [49]. Catenins exist in three isoforms α, β, and γ, which form a complex with cadherins. The wg signaling cascade is conserved in vertebrates. Ectopically expressing Wnt signaling components in *Xenopus* embryos induces axis duplication, as previously reported for *Wnt-1*[14]. Expression of *Xenopus dsh* gives phenotypes that are similar to those conferred by Wnts [50], as does dominant-negative mutants of vertebrate GSK-3 [51,52]. Ectopic expression of β-catenin [53] or plakoglobin[54] mimics Wnt-like activity. Increased cytosolic levels of catenins are thought to be critical for Wnt signaling in *Xenopus* [55].

Recently, several components of the Wnt signaling pathway, not previously identified in *Drosophila*, have been described in mouse. These include axin and APC. Axin is a protein that is structurally similar to RGS (Regulators of G-Protein Signaling) proteins and to dishevelled. Disruption of this gene in the mouse germ line causes embryonic axis duplication suggesting that it functions as a negative regulator of Wnt signaling. In fact, axin blocks Wnt-mediated axis duplication in *Xenopus*. Axin proteins that lack the RGS domain function like dominant-negative proteins as they induce axis duplication [58].

The adenomatous polyposis coli protein, APC, has been implicated in the Wnt signaling pathway based on its ability to associate with β-catenin and GSK-3 [56-58]. APC protein normally functions as a growth suppressor in colon cancer[59] as loss of APC is correlated with cancer progression. In addition, loss of APC is associated with mammary tumorigenesis in mice[60,61]. APC is thought to function by maintaining low levels of cytosolic β-catenin[62], possibly through ubiquitination of β-catenin. The APC protein itself may be regulated by GSK-3 [57]. Although APC is thought to downregulate β-catenin, a recent report demonstrated that APC expression induces axis duplication [63]. This finding is in contrast to proposed activity of APC as a suppressor of Wnt signaling and may suggest a distinct function for APC in signal transmission as well as signal suppression.

Figure 1. WNT SIGNAL TRANSDUCTION PATHWAY.



Frzb-a secreted antagonist of Wnt signaling

Frzb is a secretory polypeptide that is homologous to the frizzled family of proteins [64]. Frzb, however, has no membrane spanning domain. The region of highest homology between Frzb and frizzled is that of the cysteine rich domain. This structure suggests that Frzb functions as a secreted antagonist of frizzled receptors. In fact, Frzb expression interferes with Wnt-mediated axis duplication in *Xenopus* [65]. Frzb and Wnt proteins co-immunoprecipitate, providing direct biochemical evidence for Frzb-Wnt interactions [65]. No direct binding of Wnt to frizzled proteins has yet been demonstrated, although frizzled expression can induce cell surface association of Wnt proteins [42].

***Wnt* genes and mammary tumorigenesis**

Abnormal expression of the *Wnt*-1 gene contributes to the development of mammary tumors [66]. Transgenic mice expressing the *Wnt*-1 gene in the mammary gland develop mammary tumors with high levels of *Wnt*-1 mRNA [8]. Additional transgenic experiments also suggest Wnts cooperate with

fibroblast growth factor (FGF) members to induce mammary tumorigenesis [9,67]. Expression of the *Wnt*-1 gene in two established mammary epithelial cell lines, C57MG cells [68] or RAC311C cells [69] leads to morphological transformation from flat cuboidal cells to highly refractile, elongated cells that continue to grow post-confluence. *Wnt*-1 expression also leads to increased tumorigenicity in RAC311C cells. Thus it is well established that the *Wnt* genes are potent oncogenes in mouse mammary tumorigenesis.

Several human malignancies have been documented to have aberrant expression of *Wnt* genes. Human *WNT* genes 2, 3, 4, and 7b have been found overexpressed in breast cell lines and human breast tumors, as compared to normal breast tissue[70]. Aberrant expression of *WNT*-5a was also reported in lung, breast, and prostate carcinomas and melanomas[71].

Wnt genes in normal mammary gland development

Several *Wnt* genes are expressed during post-natal development of the mammary gland [72,73], see Table 1. *Wnt*-2, *Wnt*-4, *Wnt*-5a, *Wnt*-5b, *Wnt*-6, and *Wnt*-7a are expressed in the mammary gland during growth and differentiation (in virgin and pregnant mice) but they are not expressed in lactating glands, when the gland is no longer growing. Furthermore, the expression of several Wnt genes appears to be hormonally regulated [74]. These two findings, taken together, suggest that regulated expression of *Wnt* gene products play a role in the normal expansion or differentiation of the mammary epithelium before lactation. Several lines of evidence suggest that *Wnt* proteins may affect mammary gland development. Mice bearing a MMTV driven *Wnt*-1 transgene exhibit hormone-independent hyperplasia of mammary epithelium[8]. In these mice, the glands of both virgin females and male animals resemble those of pregnant animals. Hormone deprivation by ovarectomy has no obvious effect on the morphology of the mammary hyperplasias in *Wnt*-1 transgenics [75]. Both *Wnt*-1 and *Wnt*-3 expression can affect mammary gland growth; however, neither gene is expressed in the normal mammary gland. Thus, it has been proposed that Wnt-1 and Wnt-3 act through Wnt receptors that respond to *Wnt* family proteins normally expressed in the mammary gland. The oncogenic effects of the *Wnt*-1 and *Wnt*-3 genes may thus interfere with the normal *Wnt*-mediated regulation of mammary gland growth.

Wnt-Frizzled signaling in human carcinogenesis

Several genes involved in Wnt signal transduction, most notably β-catenin and APC, act as oncogenes or tumor suppressor genes, respectively. Mutated forms of β-catenin occur in gastric cancer cells [76] and a truncated form of β-catenin was identified as a transforming oncogene [77]. The human β-catenin gene (CTNNB1) is localized to a region of chromosome 3p21 that is implicated in tumor development [78]. Mutations in the APC gene are associated with human colon cancer. It is noteworthy that the Apc min mouse model, which has a germline APC mutation, is predisposed to develop mammary carcinomas [60,61].

The most compelling case for a role of aberrant Wnt signaling in pathobiology of human tumors has comes from studies defining APC and β-catenin mutations in colon and melanoma cancer cell lines. Colorectal tumors with intact APC genes contain activating mutations of β-catenin that alter phosphorylation sites critical to APC's ability to bind and regulate β-catenin [79]. In addition, a Tcf family member (Tcf-4), which is found to be expressed in colonic epithelium, activates transcription only when associated with β-catenin. Colon carcinoma cells devoid of APC contained a β-catenin-Tcf complex that is constitutively active [80]. Thus, constitutive transcription of Tcf target genes can be caused by loss of APC function suggesting activation of the Wnt signaling pathway is a key event in the transformation of colonic epithelium.

Genetic defects that result in regulation of β-catenin play a role in melanoma progression [62]. Abnormally high levels of β-catenin due to mis-splicing or missense mutations of the β-catenin gene were detected in several human melanoma cell lines. Other melanoma lines are missing APC or contain structurally altered APC proteins and these alterations are associated with constitutive activation of β-catenin-Tcf transcription complexes.

III. PURPOSE

The *overall objective* of the work proposed here is to determine how Wnt proteins modulate the growth of mammary epithelial cells, with the *long term goal* of understanding the role of *Wnt* genes in mammary tumorigenesis.

IV. METHODS OF APPROACH

The *overall objective* of the work proposed here is to determine how Wnt proteins modulate the growth of mammary epithelial cells, with the *long term goal* of understanding the role of *Wnt* genes in mammary tumorigenesis. Our *general strategy* will to carry out a study of the proteins encoded by ten different *Wnt* genes (*Wnt*-1, *Wnt*-2, *Wnt*-3, *Wnt*-3A, *Wnt*-4, *Wnt*-5A, *Wnt*-5B, *Wnt*-6, *Wnt*-7A, *Wnt*-7B) that will address the following *specific aims:*

1. *Examine the biochemical and secretory properties of Wnt proteins.* The coding potential for an antigenic epitope will be added to full length cDNAs encoding *Wnt*-1, *Wnt*-2, *Wnt*-3, *Wnt*-3A, *Wnt*-4, *Wnt*-5A, *Wnt*-5B, *Wnt*-6, *Wnt*-7A, and *Wnt*-7B. We will prepare stable cell lines expressing epitope-tagged Wnt proteins in order to determine how the biochemical properties of the proteins encoded by newly described *Wnt* genes compare to those described for Wnt-1 proteins. We will evaluate if these proteins enter the secretory pathway, how efficiently are they secreted, and once outside the cell are these proteins freely soluble, bound tightly to the extracellular matrix, or bound to the cell surface? Our goal is to identify Wnt proteins that can be purified for use as ligands.

2. *Determine the transforming potential of Wnt genes.* Using retroviral vectors to express the proteins encoded by these cDNAs in cultured cell lines, we will determine whether: (a) expression of these genes in cultured mammary epithelial cells leads to transformation, (b) these proteins transmit signals in a paracrine fashion, and (c) these proteins are secreted in a soluble, biologically active form.

3. *Map domains of Wnt proteins required for transforming potential.* We propose to make chimeric proteins composed of regions from active Wnt-1 proteins and regions from inactive Wnt proteins. These chimeric proteins will be used to map peptide sequences or domains of Wnt-1 that are required for biological activity. The transforming potential of the chimeric proteins will be assessed by using a rapid transformation assay, which we have recently developed. We are interested in the minimum set of sequences derived from Wnt-1 that are required for transforming potential.

4. *Characterization of Wnt proteins as ligands.* We will determine if purified Wnt proteins, act as soluble ligands to affect mammary epithelial cell physiology. Wnt proteins will be overexpressed, purified, and used to determine if they behave as agonists or antagonists of Wnt-1 activity. Biological and biochemical responses to treatment with Wnt ligands will be analyzed.

5. *Identification of Wnt-specific cell surface receptors.* Purified, active Wnt proteins will be used to probe for Wnt specific receptors on the surface of cells and to biochemically identify Wnt-specific cell surface receptors by chemical crosslinking of radiolabeled ligands. The long term goal is to identify genes encoding Wnt specific receptors.

BODY

The progress of each specific aim will described in the following Chapters.
Capital roman numerals will designate the Specific Aims (**I-IV**).
Within each aim, seperate chapters will be designated by capital letters (**A, B, ...**).
Chapters that describe a complete body of work will be presented with the following sections:
Introduction, Results, Discusssion, Materials and Methods, Figure legends, Figures.

I. Specific aim 1.
TO EXAMINE THE BIOCHEMICAL AND SECRETORY PROPERTIES OF WNT PROTEINS.

We proposed to assess the characteristics of eleven family members; including, *Wnt*-1, *Wnt*-2, *Wnt*-3, *Wnt*-3a, *Wnt*-4, *Wnt*-5a, *Wnt*-5b, *Wnt*-6, *Wnt*-7a, and *Wnt*-7b and *Wnt*-8c. Toward this goal, we have generated cDNAs encoding all eleven of these *Wnt* gene family members fused to an influenza hemagluttinin (HA) epitope to allow detection of the gene products with an anti-HA monoclonal antibody. All eleven of the HA epitope-tagged Wnt proteins have been expressed in transiently transfected 293T cells can be detected by immunoblot analysis with anti-HA antibodies. The *Wnt*-8c clone expresses protein very poorly in tissue culture cells; therefore, we continued the analysis with the other ten family members. Biochemical analysis has been initiated to determine the secretory properties of the ten different Wnt proteins. Immunofluorescence analysis carried out on Rat-1 cell lines expressing epitope-tagged Wnt proteins display prominent staining in the endoplasmic reticulum and weak staining at the cell surface. This pattern is similar to that seen for Wnt-1, indicating that the ten Wnt proteins enter the secretory pathway but are poorly secreted. Multiple forms of the various Wnt proteins are detected using immunoblot analysis, suggesting that these proteins are glycosylated. The molecular size of the different Wnt proteins correlates roughly to that predicted based upon the predicted open reading frame and potential N-linked glycosylation sites of the different Wnt genes. In conclusion, we have demonstrated that the ten Wnt proteins we have analyzed are secretory in nature and are associated with extracellular material in a fashion similar to that seen for Wnt-1. Thus, we were unable to find an efficiently secreted and highly diffusible Wnt protein family member.

II. Specific Aim 2.
TO DETERMINE THE TRANSFORMING POTENTIAL OF WNT GENES.

We have completed a study of the transforming potential of Wnt gene family members. We evaluated the transforming potential of *Wnt*-1, *Wnt*-2, *Wnt*-3, *Wnt*-3a, *Wnt*-4, *Wnt*-5a, *Wnt*-5b, *Wnt*-6, *Wnt*-7a, and *Wnt*-7b. We also compared the transfoming activity of Wnt proteins to their ability to upregulate β-catenin. This study utilized cDNAs encoding all ten of these *Wnt* gene family members fused to an HA epitope to allow detection of Wnt proteins with an anti-HA monoclonal antibody.

II. A. Transformation by Wnt Family Proteins Correlates with Regulation of β-Catenin

Introduction

The *Wnt* gene family encodes secreted factors involved in cell growth regulation, cell fate determination, organogenesis, and oncogenesis [66]. Wnt proteins have also been proposed to function as peptide growth factors within the developing mouse mammary gland, as several *Wnt* genes are expressed exclusively when the gland is in growth phase. Northern analysis of mouse mammary gland RNA at different stages of postnatal development has shown the presence of mouse Wnt-2, Wnt-4, Wnt-5a, Wnt-5b, Wnt-6, Wnt-7b, and Wnt-10b mRNAs [72-74]. In contrast, *Wnts* are not expressed in lactating mice

when the glandular epithelium has ceased proliferating. The two *Wnt* genes *Wnt*-1 and *Wnt*-3 are not normally expressed within the mouse mammary gland. *Wnt*-1 expression in adult mice is limited to round spermatids and Wnt-3 is expressed in the brain [81,82]. However, both *Wnt*-1 and *Wnt*-3 can contribute to tumorigenesis when activated by insertion of mouse mammary tumor virus (MMTV) proviral DNA in MMTV-induced mammary tumors [7,10]. Furthermore, mammary tumors arise in transgenic mice in which ectopic *Wnt*-1 is expressed from an MMTV promoter [8]. Such mice rapidly develop hyperplasia of the mammary glands prior to tumor formation, again suggesting that Wnt proteins function as peptide growth factors for mammary tissue.

Morphological transformation of cultured cells has been used to assay the biological activity of different *Wnt* gene family members. Such studies have mostly focused on the limited set of cell lines known to exhibit morphological responses to *Wnt*-1. These include C57MG mammary epithelial cells [68], RAC311 mammary cells [69,83], C3H 10T1/2 fibroblasts [84], and PC12 pheochromocytoma cells [85,86]. Ectopic expression of *Wnt*-1 in C57MG mammary epithelial cells results in elongated refractile cells which continue to proliferate beyond confluence [34,68]. Previous studies have indicated that expression of several other *Wnt* genes in C57MG cells also induced morphological transformation, while *Wnt*-4, *Wnt*-5a, and *Wnt*-6 had no effect [87,88]. In another study, C3H 10T1/2 fibroblasts responded to three different *Wnt* genes, while expression of *Wnt*-4 and *Wnt*-5b had no obvious effect [84]. These comparisons of the activity of *Wnt* cDNAs were made in the absence of Wnt protein quantification in the responsive cell lines. Since pan-specific antibodies to Wnt proteins do not exist it has been difficult to make direct comparisons of the expression levels of different Wnt proteins.

Although Wnt proteins are secretory they are predominantly associated with internal membranous components and bound to the cell surface or extracellular matrix [29-32,36,89,90]. Wingless, the *Drosophila* homologue of Wnt-1, is also associated with the cell surface and shows limited diffusion *in vivo* [91,92]. However, small amounts of biologically active Wnt-1 or Wingless can be found in culture medium conditioned by cells programmed to express these proteins [37,38].

While purified Wnt ligands have not been available for signal transduction studies, information about intracellular Wnt signaling pathways has been inferred from genetic analysis of *wingless* (wg) signaling in Drosophila. These data have lead to a generalized model of Wnt/wg signaling [40,93-95]. The candidate cell surface receptors for Wnt proteins are members of the Frizzled family of seven transmembrane proteins [42,96,97]. While details of the biochemical cascade remain unclear, the Wnt/wg signal is thought to be transduced sequentially via a Frizzled family member, one or more homologs of Drosophila Dishevelled, the kinase GSK-3, the Armadillo homologs b and g catenin, and members of the Tcf/LEF-1 family of HMG box transcription factors. The latter have been found physically associated with β–catenin in the nucleus, where they may act as transcriptional effectors of Wnt-1 signals [98]. Armadillo is required for signaling by *wingless* in Drosophila, while in *Xenopus* embryos overexpression of β–catenin is sufficient to mediate downstream effects of Wnts [49,93,99]. *Wnt*-1/*wingless* expression induces the accumulation of Armadillo/β–catenin [100-102] and this serves as a convenient and specific biochemical assay for cellular response to a Wnt-1 signal. While β–catenin levels are known to be elevated in C57MG cells transformed by Wnt-1 [102], it is not known whether this is an obligatory effect that correlates with Wnt-mediated transformation, or whether other mammalian Wnt proteins also act via pathways that regulate intracellular β–catenin levels.

To assess the role of Wnt family members in mammary cell proliferation and to investigate the specificity of intracellular signaling by Wnt proteins, we have tested the ability of ten different Wnt family proteins to transform C57MG mammary epithelial cells by both autocrine and paracrine mechanisms. By employing a set of *Wnt* cDNAs each tagged with the same epitope, we were able to relate the transforming potential of each protein to its expression level in the cells used to assess its biological activity. Our results show that *Wnt*-1, *Wnt*-2, *Wnt*-3, and *Wnt*-3a are transforming *Wnt* genes in this assay and that *Wnt*-4, *Wnt*-5a, *Wnt*-5b, and *Wnt*-7b are non-transforming. Expression of *Wnt*-6 and *Wnt*-7a induced weak morphological alterations that were observed only after extended cultivation. Analysis of intracellular β–catenin levels in the different cell lines revealed that the transforming potential of each *Wnt* gene correlated precisely with its ability to induce elevated β–catenin levels. These results support a model in which modulation of β–catenin is instrumental in Wnt-mediated transformation of mammary cells and suggest that several different Wnt proteins may activate a common intracellular signaling pathway that results in morphological transformation of these cells.

Results

Expression of Wnt family proteins in the mammary epithelial cell line C57MG.

We sought to compare the transforming potential of Wnt family proteins in C57MG cells by evaluating morphological transformation while concurrently assessing the relative expression levels of different Wnt proteins. To facilitate a direct comparison of Wnt proteins levels, we added the coding potential for a Haemagglutinin (HA) epitope to the C-terminus of ten different *Wnt* family cDNAs. We have not observed consistent qualitative differences in the transforming activities of Wnt-1 and an HA-tagged version of Wnt-1 (Wnt-1HA) in C57MG mammary epithelial cells (data not shown). Thus, placement of the HA epitope at the C-terminus does not appear to interfere with Wnt-1 function. Similar constructions were generated by placing an HA tag at the C-terminus of *Wnt*-2, *Wnt*-3, *Wnt*-3a, *Wnt*-4, *Wnt*-5a, *Wnt*-5b, *Wnt*-6, *Wnt*-7a, and *Wnt*-7b cDNAs. C57MG cell lines individually expressing each of the engineered Wnt cDNAs were generated by infection with replication-defective retroviral vectors, as described in Materials and methods.

Preliminary immunoblot analysis indicated that the Wnt-4 and Wnt-5b proteins were expressed at much lower levels than the other Wnt proteins (data not shown). To improve the expression of these proteins we substituted the *Wnt*-1 5' untranslated leader sequence for the untranslated leader sequences of *Wnt*-4 and *Wnt*-5b. Immunoblot analyses of transiently transfected cells then showed that the levels of these proteins increased to levels comparable to that of other Wnt proteins (data not shown).

Expression levels of the different Wnt proteins in C57MG cells were compared by immunoblot analyses of total cell lysates using an antibody against the HA epitope (Figure 1). The sizes and glycosylation patterns of the different Wnt proteins were consistent with the predicted amino acid sequences. Wnt proteins varied in size from 38 kd to 48 kd. One predominant species of Wnt protein was detected in cells expressing either *Wnt*-2, *Wnt*-3a, *Wnt*-4, *Wnt*-6, or *Wnt*-7a genes. Several distinct species of Wnt proteins, presumptive glycosylation variants, were observed for *Wnt*-1, *Wnt*-3, *Wnt*-5a, *Wnt*-5b, and *Wnt*-7b expressing C57MG cells. Their relative mobilities conformed to those observed in previous reports [28,89,90].

Direct transforming potential of Wnt family proteins.

To evaluate the transforming potential of *Wnt* genes we used an established assay that depends on morphological transformation of the mammary epithelial cell line C57MG. These flat cuboidal cells respond to Wnt-1 proteins by undergoing transformation to an elongated refractile morphology that is accompanied by growth post confluence to higher densities than normal C57MG cells. It is presumed that direct expression of Wnt-1 results in transformation that is dependent on both autocrine and paracrine stimulation by Wnt proteins. This assay has been referred to as a direct transformation assay [34]. Morphological criteria were used to compare the relative transforming activities of the ten different *Wnt* gene family members in this assay. Figure 2 shows that uninfected control cells and cells infected with the empty vector (LNCX) retained the flat morphology. C57MG cells programmed to express epitope-tagged *Wnt*-1, *Wnt*-2, *Wnt*-3, and *Wnt*-3a genes were morphologically transformed, as shown by their elongated refractile morphology (Figure 2). In contrast, cells expressing *Wnt*-4, *Wnt*-5a, *Wnt*-5b, *Wnt*-6, and *Wnt*-7b appeared indistinguishable from the control and did not become morphologically transformed. The appearance of C57Wnt-7a cells in monolayer culture was distinct from the control, although not radically different, suggesting that *Wnt*-7a gene expression confers a weak transforming activity (Figure 2).

The 'non-transforming' Wnt proteins were expressed at levels equivalent to that of the transforming Wnt-2 protein and in some cases were more abundant (Figure 1). This suggests that despite comparable protein levels, Wnt proteins do not have equivalent activities. All assays were done in the presence of sodium butyrate which induced a ten to twenty fold increase in Wnt protein levels compared to untreated cells and had no detectable affect on the morphology or growth properties of C57MG cells (data not shown). Assays done in the absence of sodium butyrate treatment gave comparable transformation results, despite the fact that Wnt protein expression was significantly lower (data not shown) than those presented in Figure 1.

As all *Wnt* genes described in the current study were epitope-tagged it is conceivable that these C-terminal modifications may have attenuated Wnt protein activity. We addressed this possibility by examining the activities of non-HA tagged versions of those *Wnt* genes previously classified as non-transforming or weakly transforming, namely *Wnt*-4, *Wnt*-5a, *Wnt*-5b, *Wnt*-6, *Wnt*-7a and *Wnt*-7b. We

found that C57MG expressing non-tagged *Wnt*-4, *Wnt*-5a and *Wnt*-7b showed no evidence of transformation (data not shown). However, the C57Wnt-6 and C57Wnt-7a cell lines exhibited modest changes in their morphologies after extended cultivation (data not shown). The weak transformation exhibited upon expression of Wnt-7a in C57MG was comparable to that observed in C57Wnt-7aHA cells, while the modest effect of Wnt-6 was only evident in cells expressing the untagged allele.

Paracrine Transforming Potential of Wnt family proteins.

Wnt-1 proteins have previously been shown to act as paracrine factors [33,34,37]. For example, it has been established that co-cultivation with Wnt-1-producing donor cells causes transformation of C57MG mammary cells [33,34]. To compare the paracrine transforming potential of various Wnt family proteins, we prepared a panel of non-responsive fibroblast cell lines for use as donors of Wnt activities in paracrine assays. The donor cells used here, RatB1a cells, do not display morphological or growth changes in response to Wnt-1. Retroviral vectors were used to establish ten RatB1a fibroblast populations each programmed to express a different Wnt protein. The panel included vector-infected cells, and cells expressing Wnt-1, Wnt-2, Wnt-3, Wnt-3a, Wnt-4, Wnt-5a, Wnt-5b, Wnt-6, Wnt-7a, and Wnt-7b. Wnt proteins were detected in all of these cell lines by immunoblotting (Figure 3). The relative mobilities and heterogeneity of the Wnt protein bands in RatB1a cells were mostly similar to those found for the C57MG cell lines. Ectopic expression of each of the Wnt genes in RatB1a cells did not visibly alter the morphologies of these cells (data not shown).

Co-cultivation assays were conducted to determine whether Wnt family proteins could mediate transformation in a paracrine fashion by preparing mixtures of RatB1a donor cells and C57MG target cells, as previously described [34]. C57MG cells cultivated with control Rat-B1a cells exhibited a relatively flat monolayer, while C57MG cells grown in the presence of Wnt-1, Wnt-2, Wnt-3, or Wnt-3a expressing RatB1a cells were visibly transformed (Figure 4). In contrast, RatB1a cells expressing Wnt-4, Wnt-5a, Wnt-5b, Wnt-6, Wnt-7a, and Wnt-7b did not display measurable paracrine activity in that the cell monolayers appeared indistinguishable from control cultures. By comparison of the protein levels in the donor cells, however, we can conclude that the non-transforming Wnt proteins were produced at levels comparable to several of the transforming Wnt proteins, including Wnt-2 and Wnt-3a (Figure 3).

Modulation of β–catenin by Wnt family proteins.

Having established which Wnt genes exert gross phenotypic effects on C57MG cells, we next wished to investigate their effects on intracellular β–catenin, a key component of the signaling pathway defined for Wnt-1. This allowed us to address the question of whether other members of the Wnt family may also signal via β–catenin and, if so, whether their ability to modulate β–catenin levels correlates with transforming potential. To investigate intracellular signaling induced by different Wnt proteins, we used a cell fractionation procedure to separate cytosolic and membrane-associated pools of β–catenin. This procedure identifies a cytosolic pool of β–catenin whose abundance is strongly increased in response to Wnt-1 signals (M. Giarré et al., in preparation). Immunoblotting of such fractions from C57MG cells expressing Wnt-1 showed a dramatic increase in cytosolic β–catenin relative to control cells, while the membrane-associated β–catenin levels remained constant (Figure 5). Similar fractions were analyzed from C57MG cells expressing each of the other ten *Wnt* genes. As shown in Figure 5, cells expressing Wnt-2, Wnt-3, and Wnt-3a showed elevated cytosolic β–catenin levels comparable to those expressing Wnt-1, while no increase was observed in cells expressing Wnt-4, Wnt-5a, Wnt-5b, Wnt-6, or Wnt-7b. These results show a precise correlation between a major increase in cytosolic β–catenin levels and the ability of each Wnt gene to induce robust cell transformation (summarized in Table 1). A minor increase in cytosolic β–catenin was also sometimes observed in cells expressing Wnt-7a (Figure 5). This is consistent with the weak transforming potential ascribed to Wnt-7a.

Discussion

Although *Wnt* genes were first identified as mammary oncogenes, and numerous Wnt family members have been found expressed in normal or malignant mammary tissues, the roles of mammalian Wnt proteins in cell signaling during mammary gland development are largely unknown. In addition, the intracellular signaling mechanisms stimulated by Wnt proteins other than Wnt-1 have not previously been studied in mammalian cell systems. To investigate the functional consequences of Wnt family members in mammary epithelial cells, we have tested the ability of ten different Wnt proteins to induce morphological transformation of C57MG cells by autocrine and paracrine mechanisms. In parallel, we

assayed cytosolic β–catenin levels in cells expressing each Wnt protein to determine which Wnt family members induce elevated levels of this key component of the Wnt-1 signaling pathway.

Assessment of cell transformation potential resulted in division of ten Wnt genes into distinct functional classes (Table 1). Four different *Wnt* genes, *Wnt*-1, *Wnt* -2, *Wnt* -3, and *Wnt* -3a, induced conspicuous morphological transformation when expressed in C57MG cells. The transformation resulted in uniform conversion from a flat cobblestone-like monolayer to a densely-packed culture of elongated and refractile cells. A similar phenotype has previously been described for *Wnt*-1 and *Wnt*-2 and these characteristic morphological changes are known to be accompanied by a mitogenic effect in confluent monolayers [68,87]. Since Wnt proteins are believed to act primarily as intercellular signals, we also tested their potential for transformation in a paracrine manner. For this, C57MG mammary cells were co-cultured with RatB1a fibroblasts programmed to express each of the genes. These results showed that the same four Wnt genes had clear transformation potential whether tested in autocrine or paracrine mode. In sharp contrast to these transforming Wnt genes, a second group, consisting of *Wnt*-4, *Wnt* -5a, *Wnt* -5b, and *Wnt* -7b, produced no visible changes in cell density or morphology.

Analysis of cytosolic β–catenin levels in the Wnt-expressing C57MG cell lines showed that the transforming *Wnt* genes induced elevated levels of β–catenin while the non-transforming *Wnt*s had no effect. Thus we observed a precise correlation between the ability of a *Wnt* gene to cause transformation of C57MG cells and its ability to increase steady state levels of cytosolic β–catenin. This result has several implications. First, it provides evidence that Wnt family members other than Wnt-1 may signal via β–catenin and raises the possibility that several different Wnt ligands may utilize the same signal transduction pathway. The role of β–catenin in Wnt signaling was first identified in *Drosophila*, where the β–catenin protein homolog Armadillo was found to be stabilized in response to signals from the Wnt-1 homolog Wingless [38,100]. Stabilization of an intracellular pool of β–catenin that is not complexed with adhesion molecules has also been reported in mammalian cells expressing Wnt-1, but the generality of this phenomenon to other Wnt family members has been unclear.

Current models of Wnt-1 signaling suggest that stabilization of cytoplasmic β–catenin pools leads to formation of complexes with Tcf/LEF transcription factors in the nucleus and that this may regulate downstream signals [98]. Modulation of β–catenin has also been described in colorectal cancers, in which cellular β–catenin levels can be elevated either as a result of mutations of the tumor suppressor protein APC, or by mutations in the N-terminal domain of β–catenin itself which result in increased stability [79,80]. Similar mutations in β–catenin have also been reported in melanoma cells [62]. Collectively, these data implicate β–catenin in oncogenic signaling, but the evidence linking β–catenin to Wnt-mediated cell transformation is largely indirect. By showing a precise correlation between β–catenin induction and cell transformation, our present data strengthen the notion that β–catenin plays a key role in mediating this phenotype.

Our results suggest that signals initiated by certain Wnt proteins in the mammary gland may act via the β–catenin-Tcf pathway and may therefore be potentially oncogenic if they become constitutive. In this regard it is notable that two of the *Wnt* genes that caused β–catenin induction and transformation in our assays, *Wnt*-1 and *Wnt*-3, were first identified as mammary oncogenes activated by the mouse mammary tumor virus [7,10]. The third, *Wnt*-3a, encodes a protein 90% identical to that of *Wnt*-3 [82], and the fourth, *Wnt*-2, has been found amplified and overexpressed in certain mouse mammary tumors [103]. Of these four transforming genes, *Wnt*-2 is the only one normally expressed in the mammary gland, where its message is detected at epithelial cell-stromal cell boundaries [73]. In contrast, all of the non-transforming *Wnt* genes are endogenously expressed within the mammary gland and show temporal regulation during proliferative and early lactational phases [72,74].

A potential difficulty in interpreting the absence of transformed phenotype and lack of β–catenin induction observed with *Wnt*-4, *Wnt*-5a, *Wnt*-5b, and *Wnt*-7b stems from the need to confirm that each exogenous Wnt protein had been successfully expressed in the target cells. Previous assays of Wnt gene transformation were unable to do this because of the lack of pan-Wnt antibodies to quantify protein expression [88]. To remedy this problem, we established a system in which comparisons of Wnt protein expression could be made in a uniform manner. By introducing an epitope tag into each of *Wnt* genes used in this study, the transforming activities of the genes could be evaluated in conjunction with analysis of the corresponding protein levels. Since all the C57-WntHA and RatB1a-WntHA cell lines produced detectable amounts of each epitope-tagged Wnt protein, the inability of the above four *Wnt* genes to cause transformation cannot be explained by low levels of expression. Moreover, in the case of RatB1a cells expressing Wnt-4HA, which were unable to induce transformation in paracrine assays, the same cells

were able to induce myogenesis in chick somites by paracrine stimulation in co-culture [26]. This provides confirmation that the Wnt-4HA produced by these cells was fully functional in other assays. We conclude from these results that there are clear qualitative differences among Wnt proteins with regard to their abilities to transform C57MG mammary cells.

Wnt-6 and *Wnt*-7a conferred weak phenotypic changes in C57MG monolayer cultures. For Wnt-6, this was only apparent after extended cultivation of cells expressing the untagged form of the protein. With Wnt-7a, however, subtle alterations in the morphology of C57MG cells were observed when expressing either the untagged or HA-tagged alleles, and this was reflected in a comparably weak effect on β–catenin induction. These results illustrate two points: that *Wnt*-6 and *Wnt*-7a are very much weaker transforming genes in comparison to *Wnt*-1, *Wnt*-2, *Wnt*-3 or *Wnt*-3a, and that in the case of Wnt-6, the HA tag attenuated Wnt protein activity to a marginal extent.

The transformation results presented here for Wnt-3a, Wnt-4, Wnt-5a, and Wnt-6 are broadly similar to those of Wong et al. [88], who assayed the transforming potential of Wnt cDNAs by transfection of C57MG cells. In addition, these authors reported that Wnt-5b, Wnt-7a, and Wnt-7b were capable of inducing morphological changes. However, in our experiments, Wnt-7a expression resulted in very modest transformation, while Wnt-5b and Wnt-7b showed no transforming activity and failed to increase β–catenin levels. To eliminate the possibility that the HA tag might be responsible for these differences, we also assayed transformation by unmodified Wnt alleles and obtained essentially the same results for both these genes. The reasons for the remaining discrepancies with the data of Wong et al. are therefore obscure, but may result from different methodological details in the transformation assays or potentially from differences in the subllines of C57MG cells used in the experiments.

The qualitative differences in transformation potential and β–catenin induction described here suggest that most Wnt proteins can be divided into one of two functional classes with respect to their ability to stimulate C57MG cells. In this respect, the results are consistent with the two functional groups of Wnts identified by their distinct morphogenetic effects when expressed ectopically in *Xenopus* embryos. For example, Wnt-1 induces duplication of the embryonic axis whereas Wnt-5a affects the spatial distribution of mesodermal cells [104-106]. Surprisingly, the classification of different Wnts according to their ability to cause transformation of C57MG cells or developmental defects in *Xenopus* is similar in each case, although there are several Wnts that have not been tested in both assays. These data would fit a model in which the two groups of Wnt proteins have intrinsically different functions and different signaling pathways.

An alternative explanation of the data described here is that the specificity of cellular response to a particular Wnt protein depends largely on which Wnt receptors are expressed. Consistent with this model is the recent observation that Wnt-5a induces *Xenopus* axis duplication only when co-expressed with human frizzled 5 [97]. Accordingly, C57MG cells may not express receptors for Wnt-4, Wnt-5a, Wnt-5b, or Wnt-7b, and may express receptors that bind Wnt-1, Wnt-2, Wnt-3, and Wnt-3a. Since only Wnt-2 from the latter group is found endogenously in the mammary gland, one possibility is that C57MG and other mammary epithelial cells express a receptor for Wnt-2 that is capable of binding Wnt-1, Wnt-3 and Wnt-3a. It has been suggested that Wnt-5a antagonizes the activity of Wnt-1 during cellular proliferation [107] or*Xenopus* development [108]. However, C57MG cells programmed to express Wnt-5a were not resistant to transformation in the presence of Rat-B1a expressing Wnt-1 (data not shown). Thus, our analysis provides no evidence that Wnt-5a anatagonizes Wnt-1 in cellular transformation.

With the identification of Frizzled (Fz) proteins as potential cell surface receptors for Wnt proteins [42,97], the presence or absence of particular Fz proteins in mammary cells might be informative in elucidating the specificity of Wnt protein responses. However, preliminary analysis of Fz expression in C57MG cells by RT-PCR indicates that at least 6 different Fz family members are expressed (Q. Dong, personal communication), and it therefore appears that unraveling the specific functional interactions between Wnt and Fz proteins in these cells may be complex. We anticipate that our panel of homogeneously tagged Wnt ligands will facilitate such studies and that analysis of β–catenin induction will provide a relevant assay for receptor signaling in response to Wnt ligands.

Materials and Methods

Cell Lines.

C57MG cells [109] were grown in Dulbecco's modified Eagle (DMEM) medium (4500 mg/l D-glucose) containing 10% Fetal Bovine Serum (FBS) and 10 μg/ml insulin. RatB1a cells [110] were

grown in DMEM supplemented with 7.5% Calf Serum (CS) and 2.5% FBS. The BOSC 23 retrovirus packaging cell line [111] was obtained from Warren Pear (MIT) and grown in DMEM containing 10% FBS. These cell lines were grown at 37°C in 8% CO_2.

Construction of HA Epitope -Tagged Wnt cDNAs.

cDNAs encoding Wnt proteins were obtained from several sources. The murine Wnt-1 cDNA is as previously described [27]. Human Wnt-2 cDNA was obtained from R. Wilkinson [112]. A hybrid cDNA containing N-terminal sequence of human Wnt-3 and C-terminal sequence of murine Wnt-3, and a murine Wnt-3a cDNA [82] were obtained from Henk Roelink (University of Washington)
Murine Wnt-4, Wnt-5a, Wnt-5b, Wnt-6, Wnt-7a, and Wnt-7b cDNAs [113] were kindly provided by Andrew McMahon (Harvard University).

An oligonucleotide sequence encoding the haemagglutinin (HA) antigenic determinant was appended to the 3' end of each Wnt protein coding region. These eighteen codons specified the amino acid sequence **SMAYPYDVPDYASLGPGP**, including the nine amino acid-long HA epitope, as underlined. HA-tagged Wnt cDNAs were created by subcloning each Wnt cDNA into a phagemid that contained the coding region of the HA epitope situated downstream of the newly inserted Wnt gene, separated from it by polylinker sequence. These two sequences were made co-linear by the "loop-out" mutagenesis procedure using oligonucleotides designed to eliminate the non-coding 3' sequence of the Wnt cDNA and flanking polylinker sequence. Sequences of these oligonucleotides are shown below. The 5' end of each oligonucleotide (plain text) is complimentary to the C-terminus of a specific Wnt cDNA and their 3' ends (italics) anneal to the beginning of the HA epitope-encoding sequence. Mutagenesis was accomplished using the Muta-Gene phagemid *in vitro* mutagenesis kit (Bio Rad, Richmond, CA). The presence of each fusion was confirmed by DNA sequencing.

Wnt-1HA: CGCGCGTTCTGCACGAGTGTCTA*TCCATGGCCTACCC*
Wnt-2HA: CTGACTGGACAACCGCTACA*TCCATGGCCTACCCATATG*
Wnt-3HA: CGATGTGCACACCTGCAAG*TCCATGGCCTACCCATATG*
Wnt-3aHA: CGTGCACACCTGCAAG*TCCATGGCCTACCC*
Wnt-4HA: CGTGGAGATGCACACGTGCCGG*TCCATGGCCTACCCATATG*
Wnt-5aHA: GGATCAGTTCGTGTGCAAA*TCCATGGCCTACCC*
Wnt-5bHA: GTGGACCAGTATGTCTGTAAG*TCCATGGCCTACCCATATG*
Wnt-6HA: CGCAAGGAACTCAGCCTGTGCCTC*TCCATGGCCTACCCATATG*
Wnt-7aHA:CGCACGGAGATGTATACGTGCAAG*TCCATGGCCTACCCATATG*
Wnt7bHA: CGCACCGAGGTCTTCACCTGCAAG*TCCATGGCCTACCCATATG*

The expression levels of Wnt-4 and Wnt-5b HA epitope-tagged cDNAs were increased by substituting their 5' untranslated regions with a Wnt-1 5' untranslated sequence that, in our experience, improves protein levels (unpublished observation). To do this the following oligonucleotides were used to create an NcoI site (underlined) at the initiating methionine of Wnt-4 and Wnt-5b by site-directed mutagenesis:

Wnt-4 oligo: GGCA<u>CCATGG</u>GCCCCCG
Wnt-5b oligo: CCGCTTTGGA<u>CCATGG</u>TGGTCCCAGG

NcoI fragments containing these modified cDNAs were inserted into plasmids containing the Wnt-1 5' untranslated sequence and the HA coding sequence.

Transfection of Proviral DNA and Cell line Generation.

HA-tagged cDNAs were inserted into the retroviral vector pLNCX [114] wherein neomycin phosphotransferase gene expression is controlled by the murine leukemia virus (MLV) long terminal repeat (LTR), and cDNA transcription is controlled by an internal cytomegalovirus (CMV) enhancer/promoter.

Distinct populations of C57MG cells and RatB1a cells, each expressing a unique HA-tagged Wnt cDNA, were prepared by retroviral infection. Recombinant retroviruses were generated by transiently transfecting pLNCX constructs into the BOSC 23 packaging cell line by calcium phosphate co-precipitation, as described by Pear [111]. Retroviral infection of C57MG or RatB1a cells was accomplished by co-culturing these cells and transfected packaging cells one day post-transfection as described by Brown and Dougherty [115]. Briefly, growth of transfected BOSC 23 was terminated by a four hour exposure to 10μg/ml mitomycin C (Sigma Chemical Co.). After mitomycin C treatment the packaging cell line was rinsed in phosphate buffered saline, trypsinized, and seeded with target cells into 10 cm dishes. Infections were carried out in the presence of 4 μg/ml polybrene, for 48 hours. At this time, the medium was replaced by medium containing 500 μg/ml Geneticin (GIBCO BRL Life

Technologies, Grand Island, NY). Colonies were apparent 5 days later and were pooled in medium containing 250 µg/ml Geneticin. These resultant populations, each comprised of at least 100 clones, were used in cellular and biochemical assays described here. RatB1a and C57MG cells infected by LNC-Wnt cDNAHA virus are designated RatB1a-Wnt-HA and C57Wnt-HA, respectively.

Direct Wnt Transformation Assays.

Morphological comparisons of C57Wnt-HA cell populations were made by seeding $1X10^5$-$5X10^5$ cells into each well of a 6-well plate. At 50%-80% confluence the medium was replaced by HB–CHO (Irvine Scientific, Santa Ana, CA), a defined medium that results in quiescence of non-transformed C57MG cells [34]. To maximize Wnt protein levels, the HB-CHO medium was supplemented with 1.5 mM sodium butyrate (n-butyric acid, sodium salt, Sigma Chemical Co., St. Louis, MO) to induce expression from the CMV promoter within the retroviral vector. Sodium butyrate treatment had no detectable effect on the morphology or growth properties of either C57MG cells or RatB1a cells (data not shown). Cells were photographed 3 days later on Kodak Technical Pan film.

Paracrine Wnt Transformation Assays.

To assess the morphological response of C57MG cells to exogenous Wnt stimulation, RatB1aWnt-HA cells, as stimulators, and C57MG cells, as responders, were co-cultured as described previously [36]. Again, to maximize Wnt protein expression, RatB1a cells were exposed to 1 mM sodium butyrate in normal medium 12-16 hours prior to co-cultivation. After treatment they were rinsed several times in PBS. Approximately $1X10^5$ RatB1a cells expressing Wnt proteins and approximately $2.5X10^5$ C57MG cells were co-cultivated in a 6-well dish. The following day, the medium was changed to HB-CHO. Cells were photographed 3 days later on Kodak T-Max film.

Immunoblot analysis.

Wnt-HA proteins from RatB1a and C57MG cell populations were analyzed by immunoblotting of cell lysates. To maximize Wnt-HA gene expression RatB1a or C57MG cells were treated with 1 mM sodium butyrate for 12-16 hours, conditions similar to those used in transformation assays. Cells were washed twice in cold PBS and subsequently removed from the dish in 1.5 ml cold PBS using a rubber policeman. Cells were pelleted by centrifugation at 2,000x g at 4∞C for 5 min. and lysed in 90 µl TENT buffer (20 mM Tris, pH 8.0, 2 mM EDTA, 150 mM NaCl, 1% Triton-X100) containing 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin, 2 mg/ml leupeptin, 1 mg/ml pepstatin, at 4∞C for 30 min. Lysates were then clarified by centrifugation at 10,000xg at 4∞C for 10 min, and protein contents determined using a BioRad Protein determination kit. Samples containing 40 µg protein were electrophoresed in 10% SDS-polyacrylamide gels. Proteins were transferred from gels onto nitrocellulose filters by electroblotting, and subsequently incubated overnight at 4∞C in TBST(10 mM Tris, pH 8.0, 150 mM NaCl, 0.2% Tween-20) containing 1% bovine serum albumin (fraction V). Blots were then incubated in anti-HA monoclonal antibody (12CA5, Berkeley Antibody Co., Richmond, CA) diluted 1:50 in TBST at room temperature for 2 hours after which the primary antibody was removed by washing in TBST at room temperature three times for 5 min. each. Blots were exposed to a 1:16,000 dilution of horseradish peroxidase-conjugated sheep anti-mouse immunoglobulin G (Amersham, Arlington Heights, IL). Excess secondary antibody was removed in the same manner as the primary antibody. Blots were then incubated 1-2 min in enhanced chemiluminescence detection reagents (Amersham, Inc.,) and exposed to X-ray film.

For analysis of cellular β–catenin levels, confluent cells were washed twice in TBS (10mM Tris HCl, pH 7.4, 140mM NaCl) + 2mM $CaCl_2$ and scraped from the dish in 1ml TBS containing 2mM DTT. 1mM PMSF, 0.5% aprotinin, and 2mg/ml leupeptin. The cells were homogenized by 30 strokes of a dounce homogenizer and the lysate centrifuged for 10 min at 500 x g. The crude supernatant was then fractionated at 100,000 x g for 90min at 4∞C to generate a supernatant, or cytosolic, fraction and a membrane-rich pellet fraction. Samples normalized for protein content were analyzed by gel electrophoresis and Western blotting largely as described above except that blocking was conducted in 1% non-fat dry milk in TBST, and the primary antibody was a monoclonal anti-β–catenin antibody (Transduction Laboratories, Lexington, KY) used at a dilution of 1:500. After development of the blots, equal loading of the lanes was confirmed by staining with Ponceau Red.

Tables for chapter II A.

Table 1. Summary of transforming activities of Wnt family proteins and their ability to induce cytosolic accumulation of β–catenin.

Wnt protein	Autocrine Transformation*	Paracrine Transformation	Increased β–catenin
Wnt-1	+++	+++	++
Wnt-2	+++	+++	++
Wnt-3	+++	+++	++
Wnt-3a	+++	+++	++
Wnt-4	-	-	-
Wnt-5a	-	-	-
Wnt-5b	-	-	-
Wnt-6	+	-	-
Wnt-7a	+	-	-/+
Wnt-7b	-	-	-

(- no transformation, + partial transformation, +++ complete transformation)

*Relative activities represent results of assays with C57MG cells expressing either tagged or un-tagged Wnt proteins.

Figure legends for chapter II A

Figure 1. Detection of Wnt proteins in cell lysates of retrovirus-infected C57MG populations expressing HA-tagged Wnt cDNAs. Cells were treated with sodium butyrate to induce Wnt gene expression from the butyrate-sensitive CMV promoter, as described in Materials and methods. Detergent cell lysates were prepared, fractionated through SDS-polyacrylamide gel and transferred to nitrocellulose. HA epitope-tagged Wnt proteins were detected using an anti-HA antibody. The lane labeled C57MG refers to a population of cells infected only with the LNCX vector. Positions of molecular weight protein standards are shown to the left and are in kilodaltons.

Figure 2. Morphological responses of infected C57MG epithelial cell populations expressing different HA-tagged Wnt cDNAs. Cells were seeded into six-well dishes and left to grow until 50-80% confluence whereupon the medium was replaced by HB-CHO containing 1.5 mM sodium butyrate as described in Materials and methods. Cells were photographed 3 days later.

Figure 3. Detection of Wnt proteins in cell lysates of RatB1a fibroblast populations expressing HA-tagged Wnt cDNAs. Cells were exposed to 1mM sodium butyrate for 12-16 hours and immunoblot analysis was carried out with anti-HA antibody, as described in Figure 1. The lane labeled RatB1a represents fibroblasts infected with only the LNCX vector. Positions of molecular weight protein standards are shown to the left and are in kilodaltons.

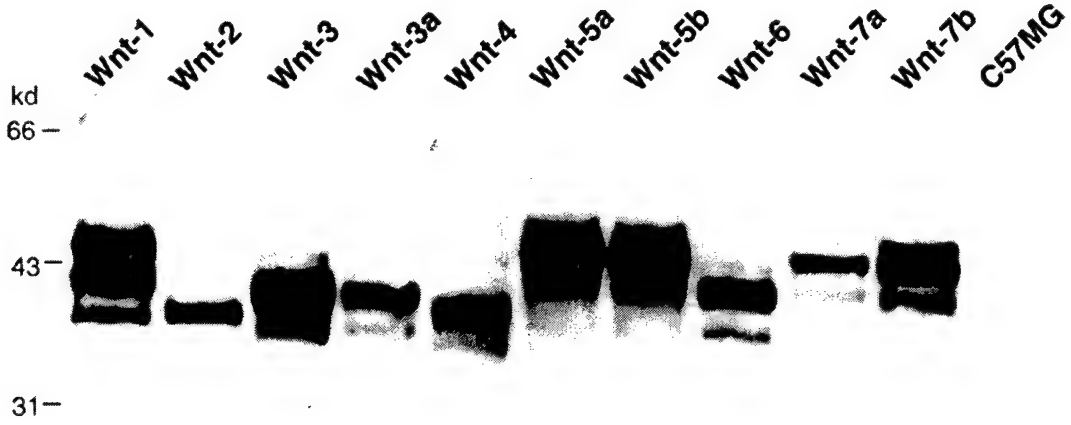
Figure 4. Morphological responses of C57MG cells co-cultured with populations of RatB1a fibroblasts expressing different HA-tagged Wnt cDNAs. C57MG cells and Wnt-producing RatB1a fibroblasts were seeded together into six well dishes as described in the Materials and methods section. After 24 hours, serum-supplemented medium was replaced by the serum-free medium HB-CHO. Cultures were photographed 3 days later. The panel designated LNCX shows C57MG cells co-cultured with control RatB1a cells infected only with the LNCX vector.

Figure 5. Modulation of cytosolic β–catenin levels by Wnt family members that cause transformation. Immunoblot analysis of β–catenin in cytosolic and membrane-rich fractions of C57MG cells exogenously expressing ten different Wnt proteins. Cell homogenates were fractionated by ultracentrifugation and normalized samples analyzed by Western blotting with a β–catenin antibody. Extracts are from cells

expressing vector alone (lanes 1 and 7), Wnt-1 (lanes 2 and 8), Wnt-2 (lane 3), Wnt-3 (lane 9), Wnt-3a (lane 10), Wnt-4 (lane 4), Wnt-5a (lane 5), Wnt-5b (lane 11), Wnt-6 (lane 12), Wnt-7a (lane 13), and Wnt-7b (lane 6). Lanes 1-6 and 7-13 are derived from two different experiments. Cytosolic β–catenin levels are strongly elevated in cells expressing Wnt-1, Wnt-2, Wnt-3, and Wnt-3a, but not in those expressing other Wnts.

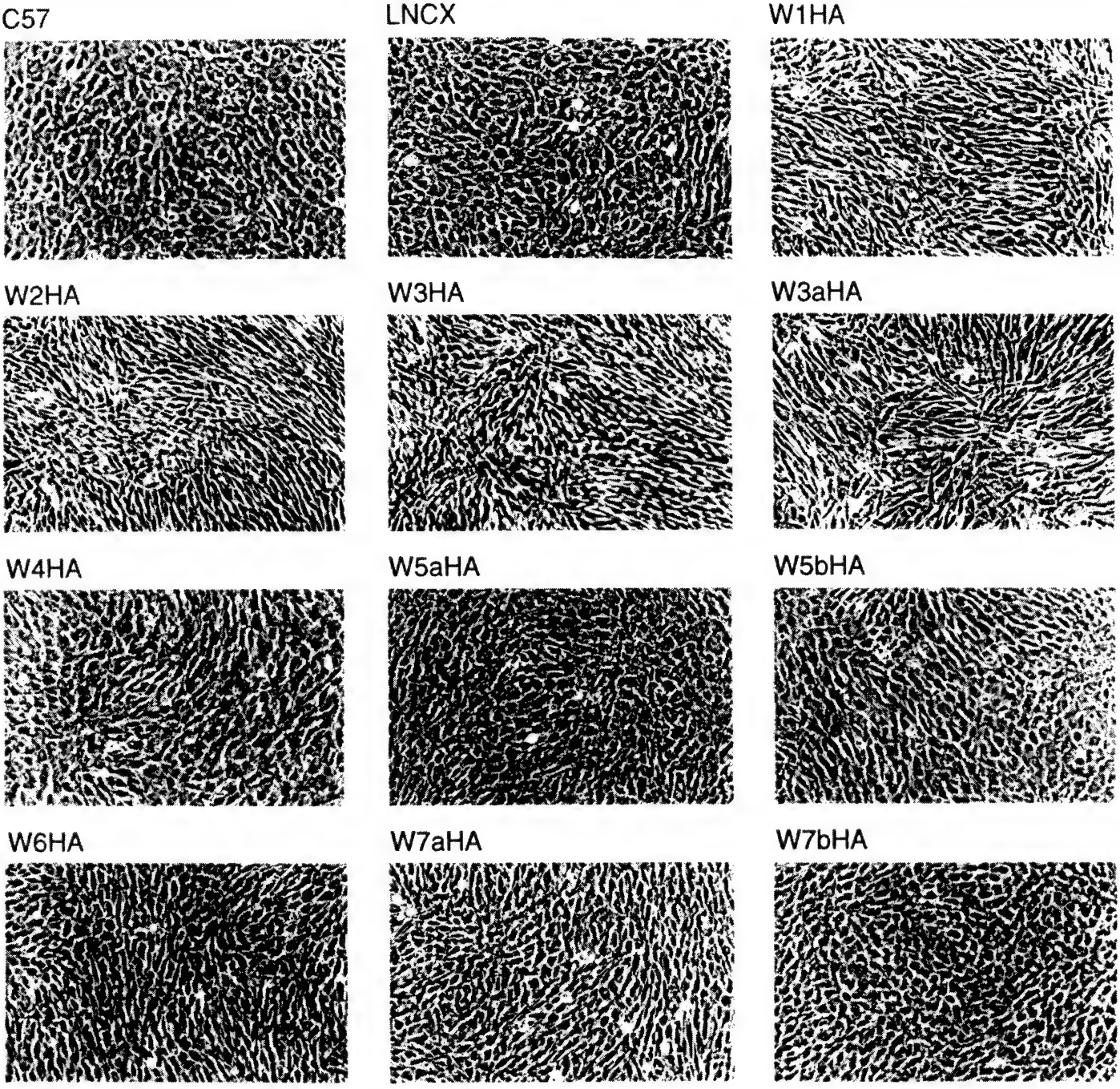
Chapter IIA

Figure 1



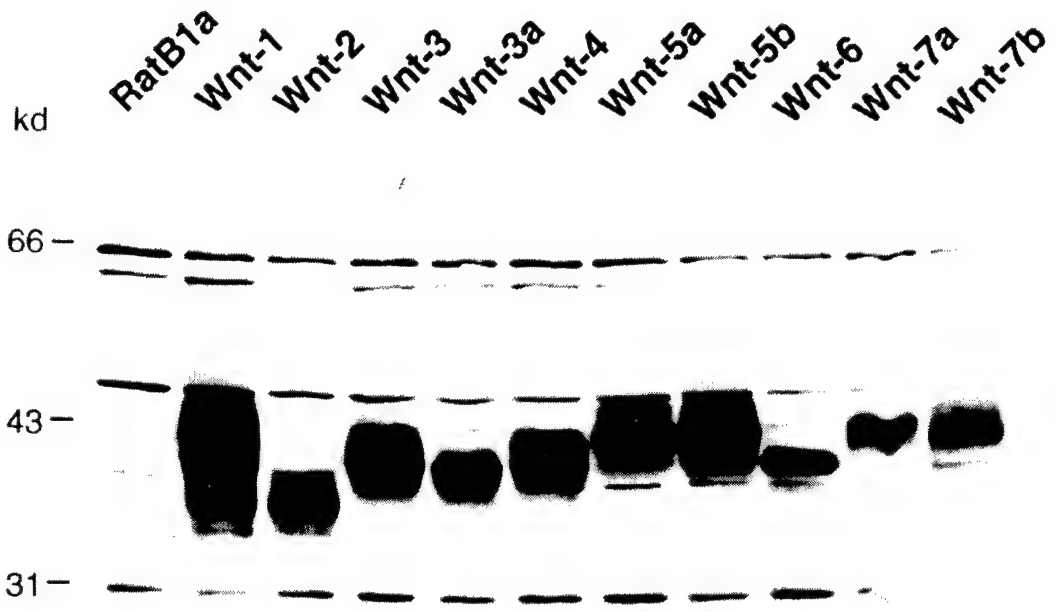
Chapter IIA

Figure 2



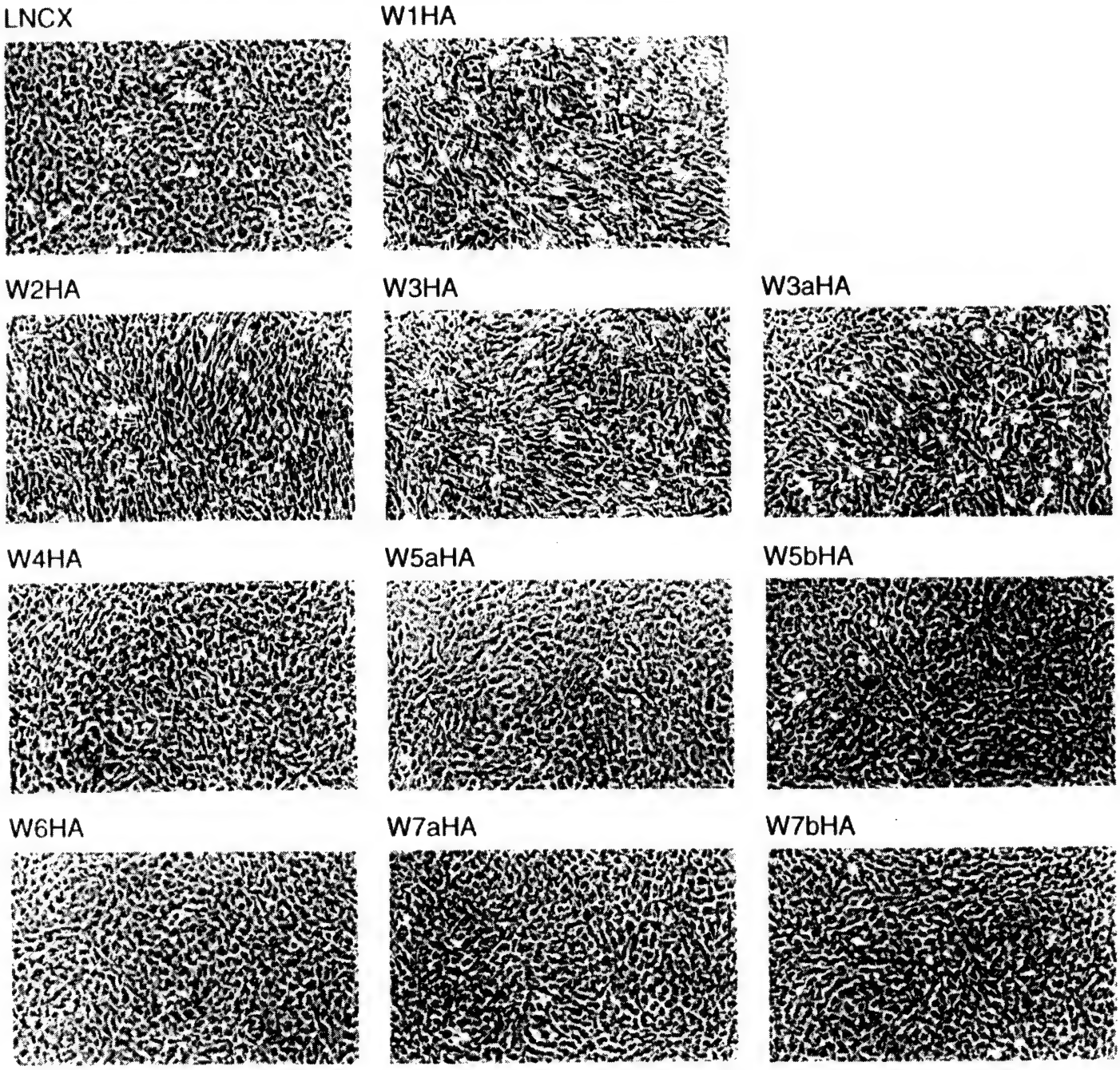
Chapter IIA

Figure 3



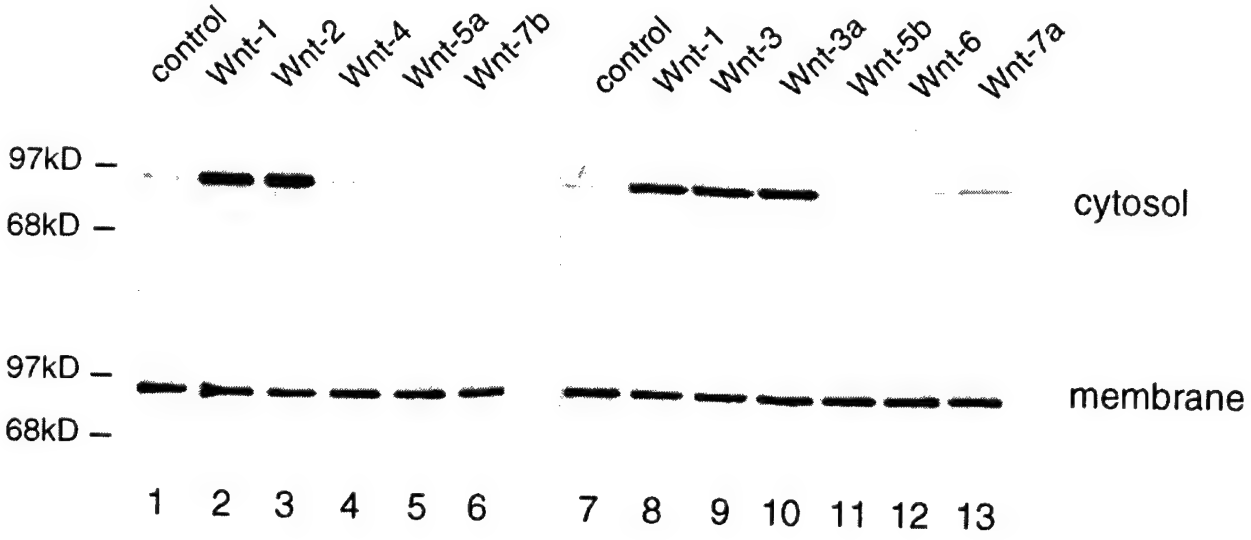
Chapter IIA

Figure 4



Chapter IIA

Figure 5



III. Specific aim 3
TO MAP DOMAINS OF WNT PROTEINS REQUIRED FOR TRANSFORMING POTENTIAL.

Little is known about the specific amino acid regions of Wnt proteins required for biological activity. Wnt proteins display anywhere from 30% to 60% amino acid identity. Conservation between different Wnts is found throughout the length of the proteins and a comparison of the sequences does not reveal highly conserved subdomains. Therefore, it is difficult to infer regions important for biological activity or regions conferring different properties among the different Wnt proteins. Identifying functional domains of Wnt proteins, therefore, must be done experimentally by modifying the structure of these proteins and testing how these modifications effect biological activity. We have documented that Wnt-1HA transforms C57MG cells whereas Wnt-5aHA does not. This observation serves as the basis to use chimeric proteins composed of regions of Wnt-1 and Wnt-5a to define the region(s) specifying transformation.

III. A. Chimeric Wnt proteins define the amino-terminus of Wnt-1 as a transformation-specific determinant.

Introduction

Wnt-1 is a member of a large gene family that functions in a myriad of processes including cell growth, cell fate determination, organogenesis, and oncogenesis [66,116,117]. Wnts are intercellular ligands that stimulate cells in vitro in an autocrine and paracrine manner [33,34,118] and act as morphogens in vivo [118-120]. The primary sequences of murine Wnt proteins are highly related sharing 40-90% amino acid sequence identity.

Wnts are secretory glycoproteins that associate mainly with the cell surface and the extracellular matrix [31,32]. The affinity of Wnts for the cell surface may be due to interactions with glycosoaminoglycans whose synthesis is required for wingless activity [121,122]. In the case of Wnt-1, cellular association may be specified by a basic region encoded at the C-terminus [123]. Wnts that are tethered to the cell surface retain paracrine activity [36,118]. Small amounts of biologically active Wnt-1 or Drosophila Wnt-1 (Wingless) appears in the culture medium of cells [37,38] but these soluble Wnt proteins have yet to be purified. N-linked glycosylation results in several distinct forms of Wnt proteins 36KDa-44KDa [28,29,34,89,90,124]. The largest forms are detectable extracellularly suggesting that highly glycosylated forms are secreted most efficiently [32,89]. However, N-linked glycosylation is not required for activity of Wnt-1 [34]. The overwhelming amount of Wnt protein is intracellular and associated with secretory vesicles [29,90] and BiP, an endoplasmic reticulum lumenal protein bound to poorly folded proteins [30]. Difficulties in secretion of Wnts may be due to the number of cysteines (22-23), some of which are known to be critical for activity [14,34].

Wnt proteins are divided into two activity groups exemplified by Wnt-1 and Wnt-5a. Wnt-1 transforms the mouse mammary epithelial cell line C57MG [88,124] and stimulates duplication of the embryonic axis in Xenopus [14]. Members of the Wnt-5a class, on the other hand, are unable to transform C57MG [88,124] or induce axis duplication although they affect Xenopus development in other ways [104,105]. Regions within Xenopus Wnt-8 and Xenopus Wnt-5a that control axis formation and inhibition, respectively, have been described [104,105].

The presumptive cell surface receptors for Wnt ligands are the frizzleds, a class of seven-pass transmembrane proteins that contain an amino terminal cysteine-rich binding domain (CRD) [42,46,97]. The CRD is also found within a group of frizzled-related secretory proteins (frps) that includes FrzB-1, an antagonist of Wnt-1 activity in Xenopus and mammalian cells [64,65,125-128].

The Wnt-1 (wingless in Drosophila) signal is thought to be transduced sequentially by a frizzled family member, orthologs of Drosophila dishevelled, glycogen synthase kinase-3, armadillo orthologs β– and γ–catenin, and members of the Tcf/LEF-1 family of transcription factors [40,93-95,129]. β–catenin plays a critical role in Wnt-1 signaling. Overexpression of β–catenin in Xenopus embryos is sufficient to recapitulate the effects of Wnt-1 [130-132] and a null mutant of armadillo resembles the wingless phenotype in Drosophila [49,93,99]. wingless, Wnt-1 and certain other Wnts induce the accumulation of

β–catenin in insect and vertebrate cells and this functions as a rapid and specific biochemical assay for cellular response to these ligands [100,101,124,127,133,134].

Morphological transformation of cultured mammalian cells is an alternate way of assaying the biological activity of different Wnt gene family members. Several cell lines are known to respond morphologically to Wnt-1 [68,69,84,134] including C57MG, a mouse mammary epithelial cell line that has a flat, cobblestone-like morphology at confluence [68]. Ectopic expression of Wnt-1 in C57MG mammary epithelial cells converts these cells by autocrine and paracrine signals to densely packed, elongated, refractile forms [34,68] that is accompanied by a mitogenic effect [68,87]. Wnt-4, Wnt-5a, and Wnt-5b, on the other hand, have no discernible affect on these cells [88,124]. There is also a direct correlation between the ability of specific Wnt family members to transform C57MG cells and their ability to induce the accumulation of cytosolic β–catenin [124]. Wnt-1, for example, induces morphological transformation and the accumulation of cytosolic β–catenin whereas Wnt-5a has no effect. Thus, modulation of cytosolic β–catenin may be instrumental in Wnt-mediated transformation of mammary cells.

To identify regions within the Wnt-1 protein that are required for transformation and signal transduction we prepared C57MG cell lines that ectopically expressed chimeric genes that contained variable amounts of Wnt-1 and Wnt-5a sequence. We found that the amino terminus was important for both transformation and cytosolic accumulation of β–catenin. Non-stimulatory chimeras that contained at least 99 amino terminal Wnt-1 residues were able to inhibit wild-type Wnt-1 stimulation of 293 cells suggesting that specificity of signaling is determined by the amino terminus.

Results

To deduce locations within Wnt-1 that control morphological transformation and the induction of cytosolic β–catenin we prepared a panel of chimeric genes that encoded variable amounts of Wnt-1 and Wnt-5a. Figure 1 is an alignment of the mouse Wnt-1 protein to the mouse Wnt-5a protein. Wnt-1 and Wnt-5a are 37% identical over the 370 amino acid residues of Wnt-1. Twenty two cysteines are located in identical positions between the two proteins. Cleavage of the Wnt-1 leader peptide is thought to occur after alanine 27 or alanine 28 [34] producing a mature Wnt-1 protein of 342 residues. We have identified amongst these two proteins six regions (I-VI) that exhibit at least 50% sequence identity. Regions of Wnt-1 were substituted for regions of Wnt-5a at locations where the two proteins are most similar (see Fig. 1 and 2). Thus, we avoided creating novel sequences at the junctions of Wnt-1 and Wnt-5a while maintaining the integrity of both conserved regions and unique regions that, presumably, control Wnt-1 specific activity.

Domains within Wnt-1 that govern morphological transformation of C57MG cells and cytosolic accumulation of β–catenin.

Regions within the Wnt-1 gene that are critical to its role as a transforming oncogene were determined using an established assay in which the mammary epithelial cell line C57MG undergoes morphological transformation in response to Wnt-1 but not Wnt-5a [88,124]. Morphological criteria were used to compare cell lines in which gene chimeras were ectopically expressed. These chimeras are shown schematically in Figure 2. Ectopic expression of the Wnt-5a gene did not alter the phenotype of C57MG cells whereas ectopic Wnt-1 gene expression converted these flat, cuboidal cells to elongated, refractile (brighter) forms that continued to grow beyond confluence (Fig. 3). The amino terminus of Wnt-1 is necessary for transformation because a chimera that contained as little as 110 residues of Wnt-5a sequence at the amino terminus was not functional (see chimera E). Chimeras F and G contained greater contributions of Wnt-5a sequence and, as expected, were not transforming. A transformed phenotype was detected when 186 amino acid residues of Wnt-1 sequence was present at the amino terminus (see chimera B). Cells expressing this chimera were more densely packed and refractile than either the control cell line or a cell line expressing chimera A where the contribution of Wnt-1 sequence is 99 residues. Thus, transformation is controlled, in part, by a domain that extends 100-186 residues from the amino terminus of Wnt-1. The transforming activity of chimera B also illustrates that the carboxy terminal 184 residues of Wnt-1 can be replaced by Wnt-5a. Although chimera B induced a change of morphology in C57MG cells it did not appear to be as strong as the wild-type Wnt-1, itself. The transformed phenotype was accentuated when the contribution of amino terminal Wnt-1 sequence to the chimera was increased. Chimeras C and D, for example, elicited transformed phenotypes that were more robust than chimera B.

Thus, the region between residues 186 and 292 augments the activity of Wnt-1 residues 1-186 in chimera B.

Figure 4a shows that, with the exception of chimera F, the non-transforming gene chimeras were expressed in C57MG cells as efficiently as those that induced transformation. The apparent molecular weights of these chimeric proteins is concordant with sizes predicted by conceptual translation of the open reading frame and potential glycosylation sites [34].

We have previously shown that transforming Wnt genes induce the accumulation of cytosolic β–catenin in C57MG [124]. Thus, Wnt-1 induces the accumulation of cytosolic β–catenin whereas Wnt-5a has no effect. Figure 5a shows that β–catenin was detected exclusively within cytosolic extracts of those chimera-expressing cell lines that displayed transformed morphologies including B, C, D. The sequence alignment of Wnt-1 and Wnt-5a in Fig. 1 shows six blocks of homology (I-VI), two of which are interrupted by regions of length variation between homology blocks III and IV and, V and VI. The influence of these length variation regions on Wnt-1 function was assessed by examining the activities of chimeras K and M wherein Wnt-1 sequences were replaced by Wnt-5a sequences at these locations (see Fig. 2). These chimeras retained the ability to effect transformation by eliciting a phenotype that was most similar to chimera B expressing cells and each induced the accumulation of cytosolic β–catenin (Fig. 5a). The results show that these length variation regions can be modified without destroying Wnt-1 activity.

Cytosolic β–catenin accumulation within 293 cells upon transient expression of gene chimeras.

We have previously shown that transient Wnt-1 gene expression within the embryonic kidney cell line, 293, induces the accumulation of cytosolic β–catenin [127]. This observation, in conjunction with the cytosolic β–catenin assay described by Shimizu et al. (1997) is a rapid way to assess Wnt-1 activity in mammalian cells and provided us with an additional means to assess structure-function relationships of these chimeras. 293 cells, like C57MG, responded to Wnt-1 but not Wnt5a (compare Figure 5a and 5b). Chimeras that failed to stimulate C57MG, such as A and E, also failed to stimulate 293 cells. Chimeras B and C, on the other hand, were able to stimulate C57MG but they failed to stimulate 293 cells. These chimeras contained less amino terminal Wnt-1 sequence than chimeras D and K and were weaker inducers of morphological transformation in C57MG cells (see Figs. 2 and 3). Among the chimeras tested, only D and K were able to elicit a response in 293. The failure of chimeras B and C to induce the accumulation of cytosolic β–catenin could not be attributed to inadequate levels of expression because all chimeric proteins were present at comparable levels (Fig. 4b). Thus, several chimeras that were active in C57MG cells were not active in 293 cells.

We took advantage of the restricted responsiveness of 293 cells to Wnt-1 stimulation by asking whether chimeras that do not function as ligands can block the ability of wild-type Wnt-1 to stimulate 293 cells. A similar experimental approach was used to demonstrate that FrzB–1 functions as an antagonist of Wnt-1 in 293 cells (Lin et al., 1997). Thus, 293 cells were co-transfected by a Wnt-1 expression vector in the presence of excess competitor expression plasmids. Figure 6a shows that Wnt-1 mediated cytosolic accumulation of β–catenin was blocked by chimeras A, B, C, M and N. These chimeras share 99 amino-terminal residues of Wnt-1 sequence. Chimeras E, G, and O, on the other hand, did not compete and these contained at least 110 amino terminal residues of Wnt-5a.

Chimera A did not stimulate C57MG or 293 cells but functioned as an inhibitor of Wnt-1 in 293 cells. The inhibitory effects of chimera A was apparent when 10 μg of competitor plasmid DNA was transfected (Figure 6b). Neither Wnt-5a or chimera G were inhibitory within the range of plasmid DNA tested. These results show that stimulation of 293 cells by Wnt-1 is controlled, in part, by a domain that lies within 99 residues of the amino terminus. The possibility that chimera A blocked Wnt-1 activity by suppressing ectopic Wnt-1 gene expression was excluded because Wnt-1 protein levels were not reduced in 293 cells that ectopically co-expressed a Wnt-1 gene and chimera A (see Fig. 6c).

Stimulation of 293 cells by Wnt-1 and Wnt-3 is blocked by a chimera that contains 99 amino terminal residues of Wnt-1

The response of C57MG cells to stimulation by Wnt-1 and Wnt-3 is detectable by cytosolic accumulation of β–catenin [124]. Figure 7 shows that 293 cells are also stimulated by Wnt-1 or Wnt-3. We asked whether the ability of Wnt-1 and Wnt-3 to stimulate 293 cells could be attributed to a common structural determinant. We demonstrated in Figure 6 that Wnt-1 activity is abrogated by a chimera that contains 99 amino terminal Wnt-1 residues whereas Wnt-5a was not inhibitory. Figure 7 shows that

chimera A also blocked Wnt-3 stimulation of 293 cells at plasmid DNA inputs of 10 and 20 micrograms whereas Wnt-5a did not inhibit Wnt-3 at 10 micrograms and partially inhibited Wnt-3 at 20 micrograms. These results, obtained in three independent experiments, suggest that Wnt-1 and Wnt- 3 are functionally similar due to a common determinant that is not found in Wnt-5a.

Discussion

We identified three domains within Wnt-1 that control its ability to induce morphological transformation of C57MG and the accumulation of cytosolic β–catenin. Three different experimental methods were used to identify and map these domains. The activities of chimeric genes in stable cell lines of C57MG were compared by assessing both morphological transformation and the accumulation of cytosolic β–catenin. The activities of chimeric genes were compared in transiently transfected 293 cells again, by detecting the accumulation of cytosolic β–catenin. These experiments were augmented by competition assays in 293 cells where the ability of Wnt-1 to induce cytosolic accumulation of β–catenin was challenged by co-transfected chimeric genes or Wnt-5a.

We found that the amino terminal 99 residues of Wnt-1 contains a determinant that distinguishes Wnt-1 from Wnt-5a. These conclusions are based on several lines of evidence summarized in Figure 2 and Table 1. Chimeras E and O contained amino terminal Wnt-5a residues and were neither active ligands or inhibitors. Chimeras A, B, C and N, on the other hand, functioned as inhibitors of Wnt-1 in 293 cells because they shared at least 99 amino terminal Wnt-1 residues (see Fig. 2). Thus, we identified a structural determinant that is unique to Wnt-1. Although the determinant contributes to ligand activity it requires a second domain, between residues 99 and 187, to promote signaling. Chimeras N and B were useful in mapping the position of this domain. Chimera N was unable to stimulate C57MG or 293 cells because it lacks Wnt-1 residues 100-186. It functioned as a competitor because it retains the amino terminal Wnt-1 specific determinant. Chimera B, on the other hand, transformed and induced the accumulation of β–catenin in C57MG because it contained residues 1-99 and Wnt-1 residues 100-186.

Other reports suggest that the amino terminus is important for function. Certain naturally occuring carboxy terminal deletion mutants of wingless are active ligands [135]. Carboxy terminal deletion mutants of mouse Wnt-1 and Xenopus Wnt-8 truncated to mouse codon 299 blocked endogenous XWnt activity [136]. Truncations beyond this point destroyed activity.

We found that a third domain appears to modulate the intensity of Wnt-1 transforming activity and the accumulation of cytosolic β–catenin. It was identified by replacing small segments of Wnt-1 with Wnt-5a, between residues 186 and 292. C57MG cells expressing chimeras K or M did not have the robust transformed phenotype of Wnt-1 cells although in each case β–catenin was detectable in the cytosol. No response to chimera M was detected in 293 cells and the response to chimera K in 293 cells was slightly reduced compared to Wnt-1. The proposed function of this domain stands in contrast to the region beyond residue 291 which was insensitve to replacement by Wnt-5a sequence. Chimera D induced morphological transformation in C57MG cells and elevation of cytosolic β–catenin in C57MG and 293 cells that was as strong as Wnt-1 itself (Fig. 2). We conclude that the carboxy terminus of Wnt-1 can be replaced by Wnt-5a without detectable loss of activity.

Mouse Wnt-1 is functionally equivalent to XWnt-8 in axis-inducing assays. Gene chimeras comprised of variable amounts of Xenopus Wnt-8 and Xenopus Wnt-5a differed in their ability to rescue axis formation in UV-ventralized embryos (Du et al.,1995). The axis-inducing activity of a chimera analogous in its design to chimera D, but using XWnt-8 instead of Wnt-1, was active. However, a chimera that was structurally equivalent to chimera C was inactive. Thus, axis inducing activity is controlled by the carboxy terminus of XWnt-8. We found that morphological transformation of C57MG and the accumulation of cytosolic β–catenin is controlled by amino terminal rather than carboxy terminal residues (see Fig. 2). This conclusion is supported by several lines of evidence. We found that biological activity was retained by chimeras B and C in which 171-184 residues of carboxy terminal Wnt-1 sequence was replaced by Wnt-5a sequence. Furthermore, sequence replacements in a sub-carboxy terminal region, between residues 186 and 292, did not destroy activity (chimeras K and M). We also found that Wnt-1 stimulation of 293 cells was blocked specifically by chimeras that contained amino terminal Wnt-1 sequence. Different Wnt protein domains may be required for Xenopus axis formation and morphological transformation of C57MG, accounting for what appear to be differences in these two studies. Additionally, the two chimera sets included different Wnt proteins, Wnt-1 and Wnt-5a on one hand and XWnt-8 and XWnt-5a on the other hand.

We observed that chimeras whose activities were compromised in C57MG were comparably reduced in 293 or were inactive. The differential response to Wnt-1 stimulation by C57MG and 293 may be due to the expression of different receptor(s) for Wnt-1 or intrinsic differences within the intracellular part of the signaling pathway.

In this study we have described the use of Wnt chimeras as competitors to locate regions within the Wnt-1 ligand that control signaling. Because purified Wnt ligands are not available to conduct classical receptor-binding assays we have adapted transient co-transfection assays to study ligand specificity. Again, only chimeras that contained Wnt-1 sequence at the amino terminus blocked Wnt-1 activity. We also found that Wnt-3 stimulation of 293 cells could be blocked by a chimera that contained 99 amino terminal Wnt-1 residues. The simplest interpretation of this result is that Wnt-1 and Wnt-3 are structurally similar, allowing them to interact with an extracellular component of the signaling pathway that is common to both ligands. This component could be a Wnt receptor or a requisite co-factor. It is possible that the amino terminal 99 residues of Wnt-1 contains a determinant that allows a receptor (or co-factor) to discriminate between Wnt-1 and other Wnt ligands (Wnt-5a). The second domain, between residues 99 and 187, might interact with the amino terminus of Wnt-1 to facilitate its presentation to the receptor.

Materials and methods

Preparation of chimeric Wnt genes

Hemaglutinin (HA)-tagged versions of Wnt-1 and Wnt-5a cDNAs in pBluescript are described in [124]. Eleven chimeric cDNAs containing variable amounts of Wnt-1 and Wnt-5a were prepared by the method of strand overlap extension [137] using these plasmids as PCR templates and restriction endonuclease digestion and ligation (see Figures 1 and 2). Points of fusion between these two Wnt cDNAs occurred in regions of amino acid sequence identity so as not to disturb protein secondary structure (see Fig. 1). PCR products were synthesized using Pfu polymerase (Stratagene, Inc., Torrey Pines, CA). Pilot experiments were conducted for each pair of primers to determine conditions that promoted maximum yield and specificity by altering magnesium ion concentration, annealing temperature, and cycle number. The integrity of all PCR products was assessed by mapping the location of restriction endonuclease sites that distinguished Wnt-1, Wnt-5a and chimeric PCR products. Full-length chimeric cDNAs were assembled by annealing PCR products, previously purified by gel electrophoresis, and amplifying them with primers complementary to pBluescript vector sequences that flanked the polylinker. All chimeric cDNAs were cloned into the pZNCX retrovirus vector described below.

Cell Lines

C57MG cells [109] were grown in Dulbecco's modified Eagle (DMEM) medium (4500 mg/l D-glucose) containing 10% Fetal Bovine Serum (FBS) and 10 µg/ml insulin. The 293-derived retrovirus packaging cell line, Bosc23 [111] ,was obtained from Warren Pear (MIT) and grown in DMEM containing 10% FBS. We refer to this line as 293. These cell lines were grown at 37°C in 8% CO_2.

Transfection of Proviral DNA into Bosc23 and Cell line Generation

HA-tagged Wnt-1, Wnt-5a , and chimeric cDNAs or a nuclear-localizing form of the lacZ gene (LZ^n)[138] were inserted into pZNCX, a retroviral vector of our own design, wherein cDNA transcription and neomycin phosphotransferase gene expression is controlled by an internal cytomegalovirus (CMV) enhancer/promoter.

Distinct populations of C57MG cells, each expressing a unique HA-tagged Wnt cDNA chimera, were prepared by retroviral infection. Two sets of chimeric Wnt-expressing cell populations were prepared and examined independently. Recombinant retroviruses were produced by transiently transfecting recombinant ZNCX constructs into the Bosc23 packaging cell line by calcium phosphate co-precipitation, as described by Pear et al (1993)[111]. Retroviral infection of C57MG cells was accomplished by co-culturing these cells and transfected packaging cells one day post-transfection as described [115]. Briefly, growth of transfected Bosc23 was terminated by a four hour exposure to 10 µg/ml mitomycin C (Sigma Chemical Co., MO.). After mitomycin C treatment the packaging cell line was rinsed in phosphate buffered saline (PBS), trypsinized, and seeded with target cells into 10 cm dishes. Infections were carried out in the presence of 4 µg/ml polybrene, for 48 hours. At this time, the medium was replaced by medium containing 500 µg/ml Geneticin (GIBCO BRL Life Technologies, Grand Island,

NY). Colonies were apparent 5 days later and were pooled in medium containing 250 μg/ml Geneticin. These resultant populations, each comprised of at least 100 clones, were used in cellular and biochemical assays described here.

Direct Wnt Transformation Assays

Morphological comparisons of C57Wnt-HA cDNA chimera cell populations were made by seeding $1X10^5$-$5X10^5$ cells into each well of a 6-well plate. At 50%-80% confluence the medium was replaced by HB-CHO (Irvine Scientific, Santa Ana, CA), a defined medium that results in quiescence of non-transformed C57MG cells [34]. To maximize Wnt protein levels, the HB-CHO medium was supplemented with 1.5 mM sodium butyrate (n-butyric acid, sodium salt, Sigma Chemical Co., St. Louis, MO) to induce expression from the CMV promoter within the retroviral vector. Sodium butyrate treatment has no detectable affect on the morphology or growth properties of C57MG cells in this assay [124]. Cells were photographed on Kodak Technical Pan film which was developed in a small tank containing Kodak HC-110 developer (dilution B) for eight minutes.

Inhibition of Wnt-1 induced cytosolic accumulation of β–catenin in 293 cells by transient co-transfection of Wnt-1 and competitor plasmids

293 cells, grown in 10 cm dishes, were transiently transfected according to the method of Pear et al (1993)[111] by a plasmid cocktail containing a CMV-promoter driven Wnt-1 expression vector in the presence of competitor plasmid or carrier plasmid containing a nuclear-localizing form of the lacZ gene ($pZNCLZ^n$). Cells were harvested one to two days post-transfection and cytosolic extracts were prepared and analyzed for the presence of β–catenin as described below.

Immunoblot analyses of Wnt-HA proteins

Wnt-HA proteins from 293 and C57MG cell populations were analyzed by immunoblotting cell lysates. To maximize Wnt-HA gene expression in C57MG cells, tissue culture medium was supplemented with 1 mM sodium butyrate for 12-16 hours, conditions similar to those in transformation assays described above [124]. Cells were washed three times in cold PBS and subsequently removed from the dish in 1 ml cold PBS using a rubber policeman. Cells were pelleted by centrifugation at 2,000x g at 4°C for 5 min. and lysed in 90 μl TENT buffer (20 mM Tris, pH 8.0, 2 mM EDTA, 150 mM NaCl, 1% Triton-X100) containing 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, at 4°C for 30 min. Lysates were clarified by centrifugation at 10,000xg at 4°C for 10 min, and protein contents determined using a BioRad Protein determination kit. Samples containing 40 μg protein were electrophoresed in 10% polyacrylamide-SDS gels. Proteins were transferred from gels onto nitrocellulose filters by electroblotting, and subsequently incubated overnight at 4°C in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 0.2% Tween-20) containing 1% bovine serum albumin (BSA, fraction V). Wnt-HA proteins were detected by incubating the appropriate blots in anti-HA monoclonal antibody (12CA5, Berkeley Antibody Co., Richmond, CA) diluted 1:100 in TBST-1% BSA at room temperature for 2 hours after which the primary antibody was removed by washing in TBST at room temperature three times, 5 min. each. Blots were exposed to a 1:16,000 dilution of horseradish peroxidase-conjugated sheep anti-mouse immunoglobulin G (Amersham, Arlington Heights, IL). After 1 hour excess secondary antibody was removed as described for the primary antibody. Blots were then incubated 1-2 min in enhanced chemiluminescence detection reagents (Amersham, Inc., Rockford, IL) and exposed to X-ray film.

Detection of cytosolic β–catenin in transiently transfected 293 cells and C57MG cells

Cytosolic and membrane preparations of transfected 293 and C57MG cells were made essentially as described by Shimizu et al., (1997) with slight modifications. Confluent cells, in 10 cm dishes, were washed three times in PBS and scraped into 0.75-1ml TBS (10mM Tris HCl, pH 7.4, 140mM NaCl) containing 2mM DTT 1mM PMSF, 2 μg/mL aprotinin, and 1μg/mL leupeptin. The cells were homogenized by 30 strokes of a Potter-Elvejhem homogenizer and the lysate centrifuged 10 min at 500 x g. The crude supernatant was re-centrifuged in a Beckman SW50.1 rotor at 100,000 x g, 90min at 4°C to generate a supernatant, or cytosolic, fraction and a membrane-rich pellet. Samples containing 40 μg protein were electrophoresed through 8% polyacrylamide-SDS gels, blotted onto Immobilon P (Millipore, Inc., Bedford, MA) and probed by antibodies as described above except that the primary antibody was a monoclonal anti-β–catenin antibody (Transduction Laboratories, Lexington, KY) used at a dilution of 1:2500-1:5000. Blots were incubated 5 min in enhanced chemiluminescence detection reagents (Super Signal, Pierce, Rockford, IL.) that were diluted in an equal volume of water immediately before use.

Retrovirus-infected C57MG cell lines were stimulated in the presence of 1.5 mM sodium butyrate approximately 16 hours prior to harvesting (Shimizu et al., 1997).

Figure Legends for chapter III A.

Figure 1. Amino acid sequence alignment of mouse Wnt-1 and Wnt-5a. Gaps are represented by dashes. Homology regions I-VI (underlined twice) are 15-53 residues in length and exhibit ≥ 50% sequence identity (*) between Wnt-1 and Wnt-5a. The locations of junctions between Wnt-1 (in bold numbers) and Wnt-5a sequences (plain text) within chimeras are shown above the Wnt-1 sequence. Residue positions at the end of each line are shown for Wnt-1 (bold) and Wnt-5a (plain text). Diamonds (◊◊) indicate presumptive signal peptidase cleavage sites within Wnt-1 [34]. The leader peptide cleavage site within Wnt-5a is not known. An alignment of murine Wnt-1 to additional mammalian Wnt proteins is found in [113].

Figure 2. A description of Wnt cDNAs that are chimeric for Wnt-1 and Wnt-5a. The contribution of Wnt-1 (shaded region) and Wnt-5a (light region) to each chimera is indicated. Due to internal variations in length between the two proteins the Wnt-5a sequence appears disproportionately represented by 10 residues. The abilities of chimeras to effect morphological transformation and the accumulation of cytosolic β–catenin in C57MG is summarized to the right side of each chimera. Chimera N and O were not transforming (data not shown). The specific contribution of Wnt-1 and Wnt-5a codons to each chimeric cDNA is as follows: A)W1:1-99/W5a:111-379, B)W1:1-186/W5a:198-379, C)W1:1-199/W5a:219-379, D)W1:1-291/W5a:301-379, E)W5a:1-110/W1:101-370, F)W5a:1-197/W1:187-370, G)W5a:1-300/W1:292-370, K)W1:1-186/W5a:198-219/W1:200-370, M)W1:1-252:W5a:272-300:W1:292-370, N)W1:1-99/W5a:111-217/W1:198-370, O)W5a:1-110/W1:101-196/W5a:217-379.

Figure 3. Morphological responses of C57MG epithelial cell populations expressing HA-tagged Wnt cDNA chimeras. Cells were seeded into six-well dishes and left to grow until 50-80% confluence when the medium was replaced by HB-CHO containing 1.5 mM sodium butyrate as described in Materials and methods. Cells were photographed 3 days later. Control refers to a population of cells infected by retrovirus containing a Wnt-gene chimera that was not expressed. Each set of chimeric Wnt-expressing cell populations were assayed at least twice (see Materials and methods).

Figure 4. Detection of Wnt protein chimeras in cell lysates of C57MG or 293 cells expressing HA-tagged Wnt cDNA chimeras. Wnt proteins were detected using an anti-HA antibody as described in Materials and methods. Positions of molecular weight protein standards are shown to the right in kilodaltons. (a) Wnt protein chimeras in C57MG cell populations. Control refers to a population of cells that were infected by a retrovirus containing a non-expressing Wnt gene chimera. Protein expression was assayed in both sets of cell populations (see Materials and methods). (b) Wnt protein chimeras in cell lysates of transiently transfected populations of 293 cells. Control refers to 293 cells that were transfected by pZNCLZ^{n}. The apparent molecular weight distribution of ectopically produced Wnt proteins in 293 differs from C57MG, presumably due to incomplete glycosylation [34].

Figure 5. Detection of cytosolic β–catenin in C57MG and 293 cells that express Wnt gene chimeras. Cells were homogenized in isotonic buffer and centrifuged at 100,000xg. Supernatant (cytosol)-derived proteins were electrophoresed, blotted, and exposed to anti-β–catenin antibody as described in Materials and methods. (a) Lysates obtained from C57MG epithelial cell populations expressing different HA-tagged Wnt cDNA chimeras were examined for the presence of cytosolic β–catenin. Control refers to a population of cells infected by retrovirus containing a Wnt-gene chimera that was not expressed. Each cell population was examined at least twice and comparable results were obtained. (b) Lysates obtained from 293 cells transiently transfected by HA-tagged Wnt cDNA chimeras were examined for the presence of cytosolic β–catenin. Control refers to cells transfected by pZNCLZ^{n}. Transient transfection experiments of each chimera were performed multiple times with comparable results.

Figure 6. Wnt-1 mediated cytosolic accumulation of β–catenin in 293 cells is blocked by co-expression of Wnt-HA cDNA chimeras. 293 cells were transiently transfected by 0.5 μg Wnt-1 gene expression vector and 30 μg competitor plasmid or the control (carrier) plasmid pZNCLZ^{n}. Cytosolic extracts were prepared two days later and analyzed for the presence of β–catenin as described in Materials and methods. Identical results were obtained from two independent experiments. (a) β–catenin expression was detected in 293 cells co-transfected by Wnt-1 and competitor plasmids, as indicated. The far-left lane depicts β–catenin levels in cells transfected by carrier (pZNCLZ^{n}) DNA exclusively. (b) β–catenin expression was detected in 293 cells co-transfected by Wnt-1 and variable amounts of competitor plasmid, as indicated. The far-left lane depicts β–catenin levels in cells transfected by pZNCLZ^{n} DNA exclusively. (c) Ectopic Wnt-1 gene expression was detected in 293 cells co-transfected by an excess of chimera A plasmid. Pellet (membrane)-derived proteins were electrophoresed, blotted and exposed to Mc123, a monoclonal antibody that binds a determinant present in wild type Wnt-1 but not chimera A [28]. The far-right lane is a control for antibody binding.

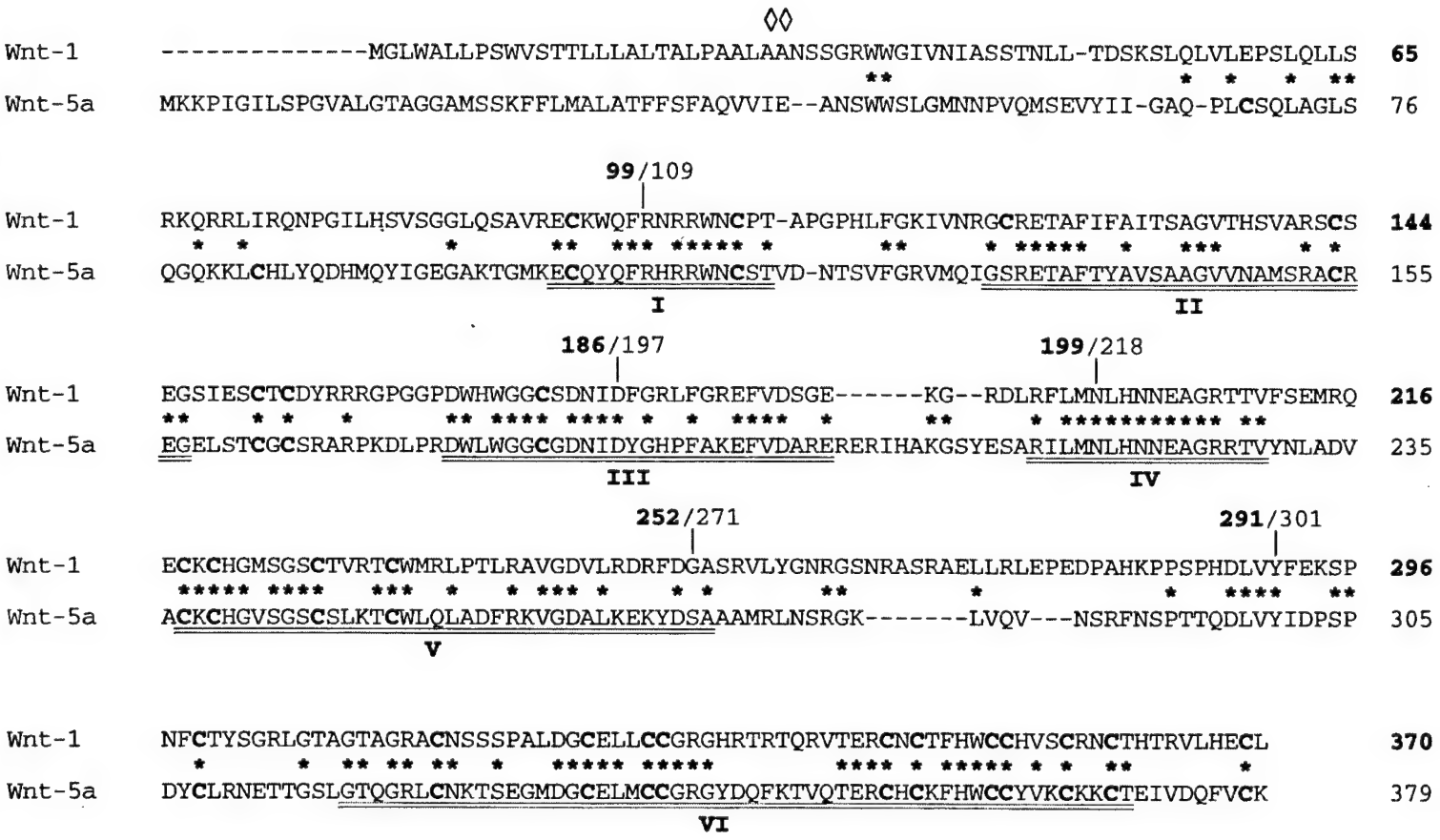
Figure 7. Chimera A blocks the ability of Wnt-3 to stimulate the accumulation of cytosolic β–catenin in 293 cells. (a) 293 cells were transiently transfected by 1 μg Wnt-1 or Wnt-3 gene expression vector and 0, 10, or 20 μg competitor or control plasmid (pZNCLZ^{n}) which was also added as carrier to achieve 20 μg plasmid DNA per transfection. Two days later cytosolic extracts were prepared and analyzed for the presence of β–catenin as described in Materials and methods. Three experiments, performed independently, produced identical results.

Legend to Table 1 for chapter III A.

Summarized here are data taken from Figs. 5 and 6. 293 cells were transiently transfected by constructs shown above and scored for their ability to either stimulate the accumulation of cytosolic β–catenin or act as dominant-negative mutants by inhibiting Wnt-1 activity.
NA, not applicable.

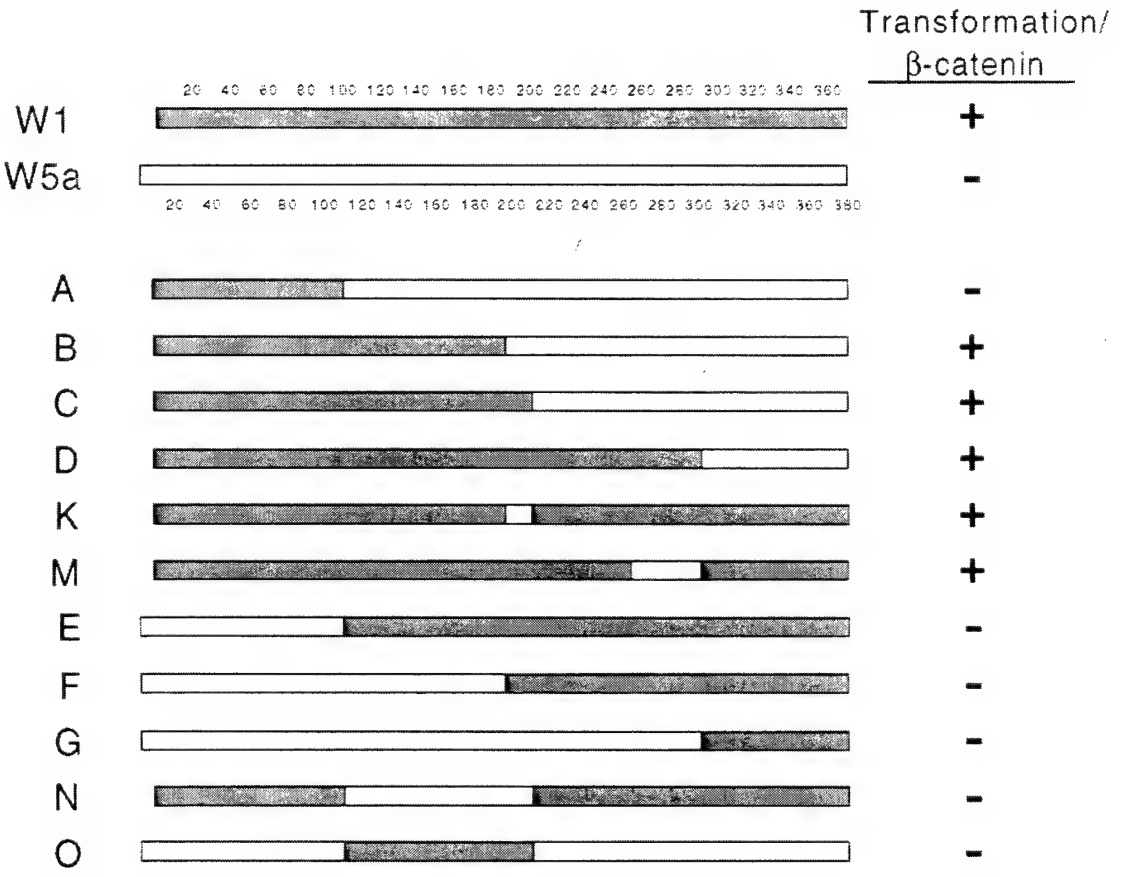
Chapter IIIA

Figure 1



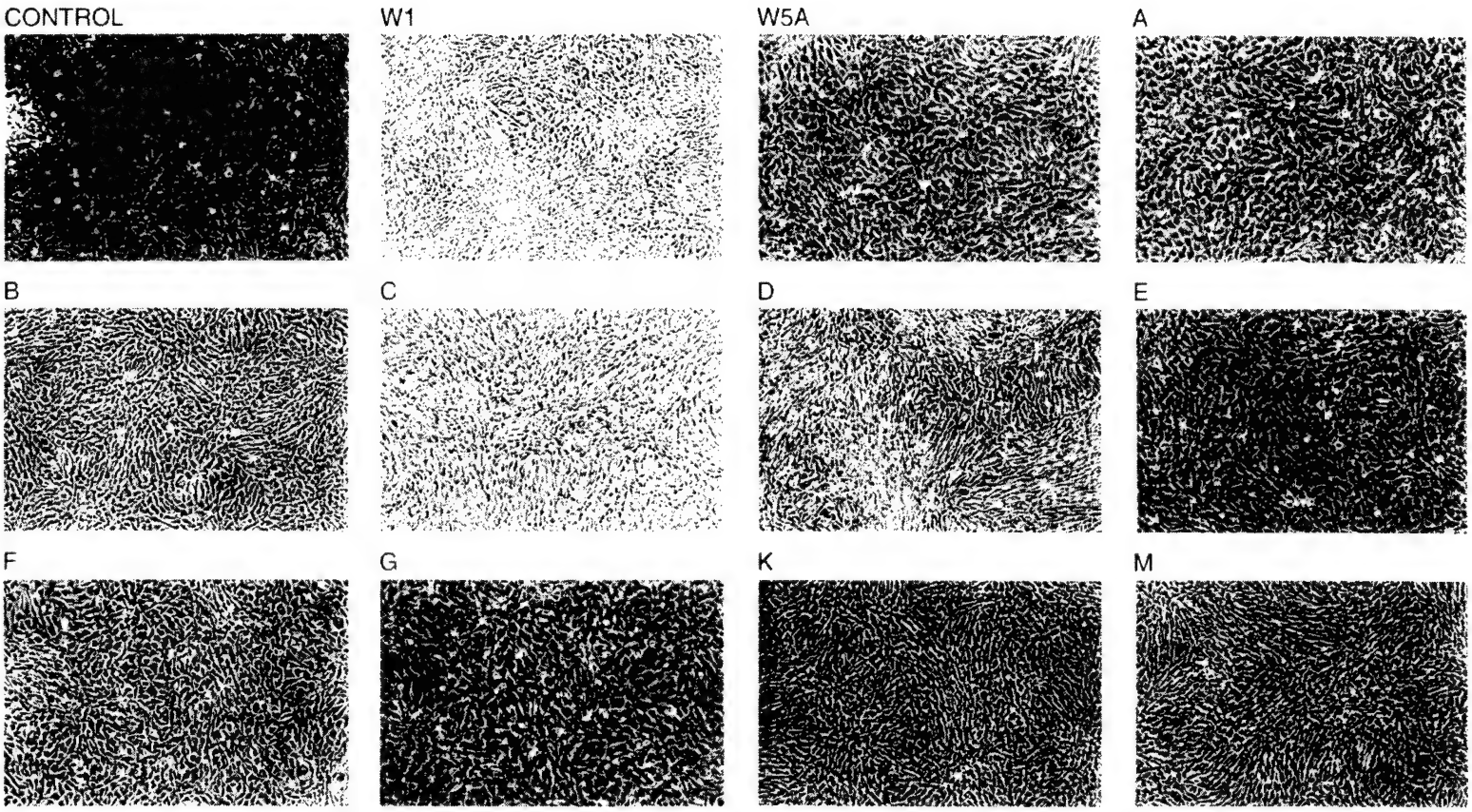
Chapter IIIA

Figure 2



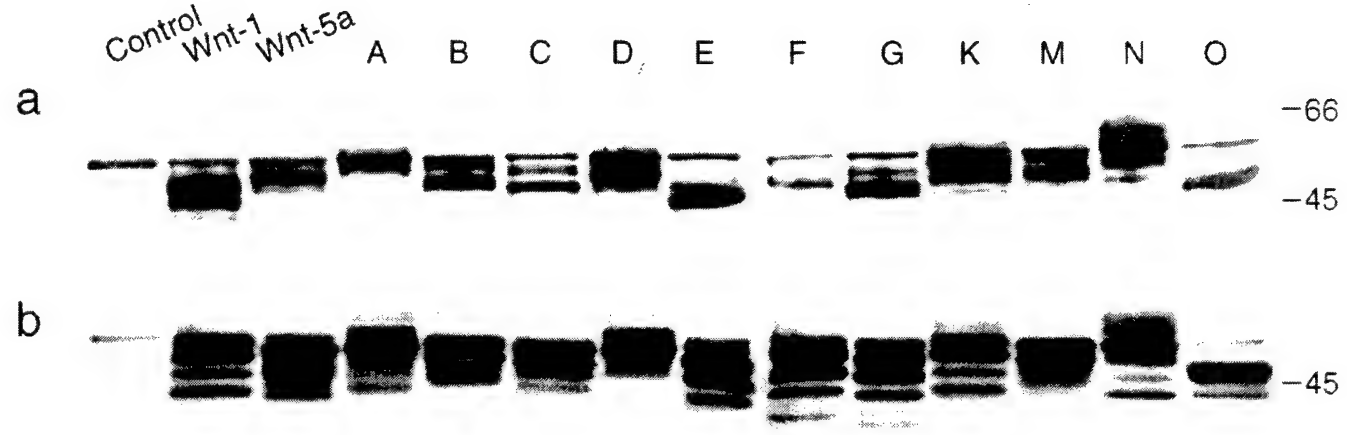
Chapter IIIA

Figure 3



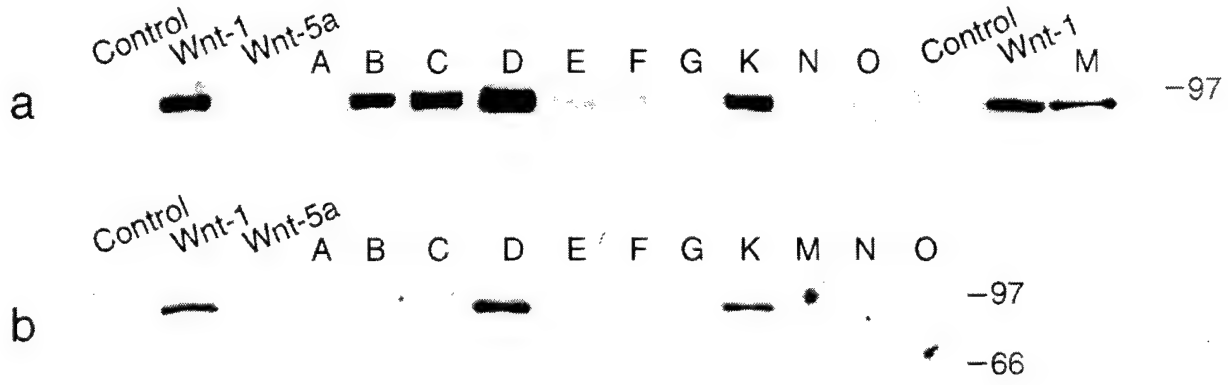
Chapter IIIA

Figure 4



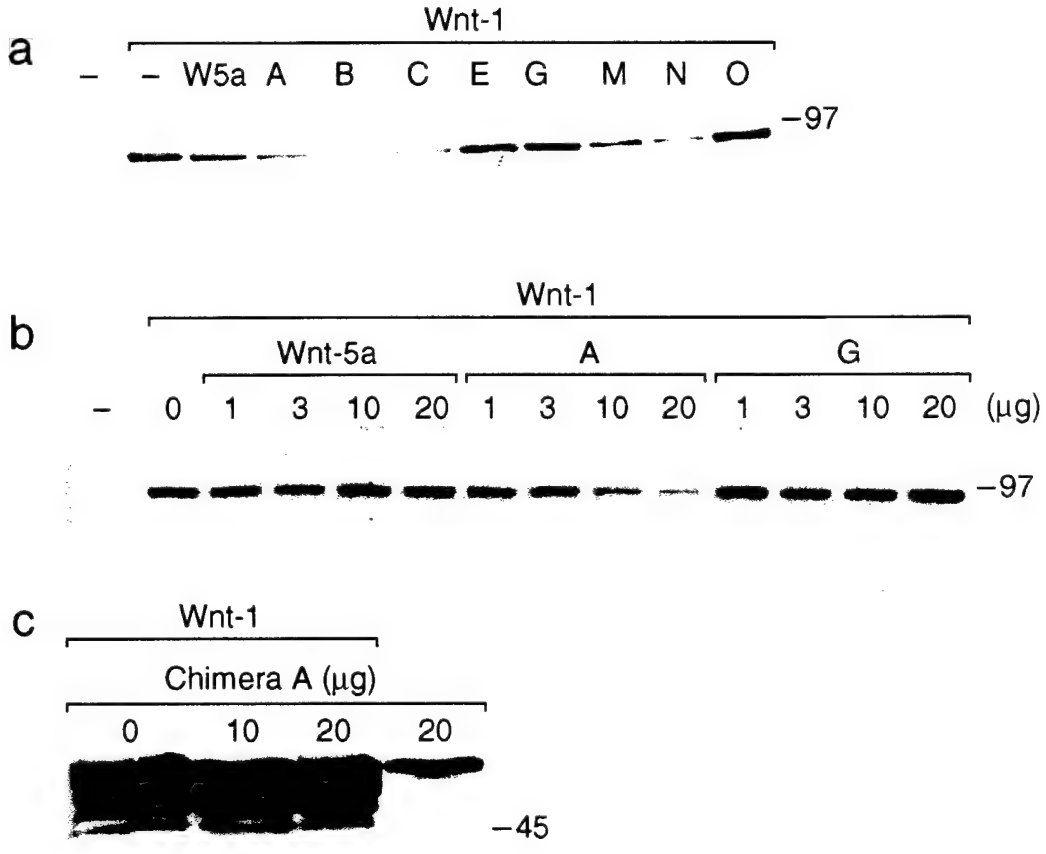
Chapter IIIA

Figure 5



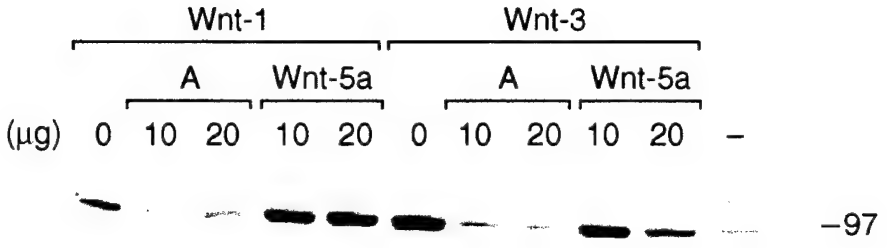
Chapter IIIA

Figure 6



Chapter IIIA

Figure 7



Chapter IIIA

Table 1

Table 1 Activities of chimeras in 293

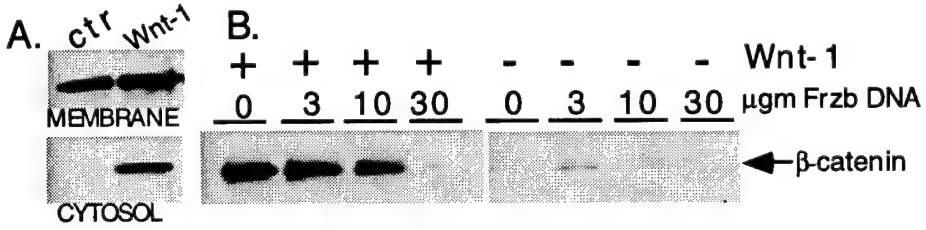
Wnt construct	*β-catenin accumulation in cytosol*	
	Stimulatory	*Inhibitory*
W1	+	NA
W5a	-	-
A	-	+
B	-	+
C	-	+
D	+	NA
K	+	NA
M	-	+
E	-	-
F	-	-
G	-	-
N	-	+
O	-	-

IV.

Specific aim 4. *CHARACTERIZATION OF WNT PROTEINS AS LIGANDS.*
Specific aim 5. *IDENTIFICATION OF WNT-SPECIFIC CELL SURFACE RECEPTORS.*

These aims were originally focused on the eventual identification of Wnt specific receptors. Recently, the Frizzled family of proteins have been identified as Wnt signaling receptors. These proteins are characterized by a cysteine rich extracellular domain and seven membrane spanning segments. No group has yet demonstrated that frizzled proteins bind directly to Wnt proteins. In collaboration with Dr. Frank Luyten, we have recently shown that Wnt-1 will bind to a frizzled-like protein, Frzb-1. To take advantage of this finding we propose to focus these aims on the interaction between Frzb and Wnt as a means of analyzing receptor interaction with Wnt proteins. The following section describes progress made in this area.

Frzb-1 contains an N-terminal domain with 50% identity to the cysteine-rich domain (CRD) of *Drosophila* frizzled [64], proposed to be the ligand binding domain. In fact, the frizzled CRDs are as homologous to the CRD of frzb as they are to each other. Recently, the Luyten laboratory found that Frzb-1 is a secreted antagonist of Wnt-8 mediated axis duplication in *Xenopus* [65]. We investigated if the inhibitory effect of Frzb-1 on Wnt-1 signaling, as observed in *Xenopus*, could also be demonstrated in mammalian cells. Ectopic Wnt-1 expression in 293 cells induces the accumulation of ß-catenin within the cytosol, whereas membrane-associated levels of ß-catenin remain virtually unchanged (Fig. 1A). Figure 1B shows that the induction of cytosolic ß-catenin by Wnt-1 is attenuated in the presence of increasing amounts of Frzb-1 encoding plasmid. Frzb-1 expression alone had no affect on cytosolic ß-catenin levels. This data is described in detail in the Appendix paper (Lin *et.al.*) where we also demonstrate that the CRD domain of Frzb-1 is required for Wnt-1 binding and that both Wnt-1 and Wnt-5a bind Frzb.

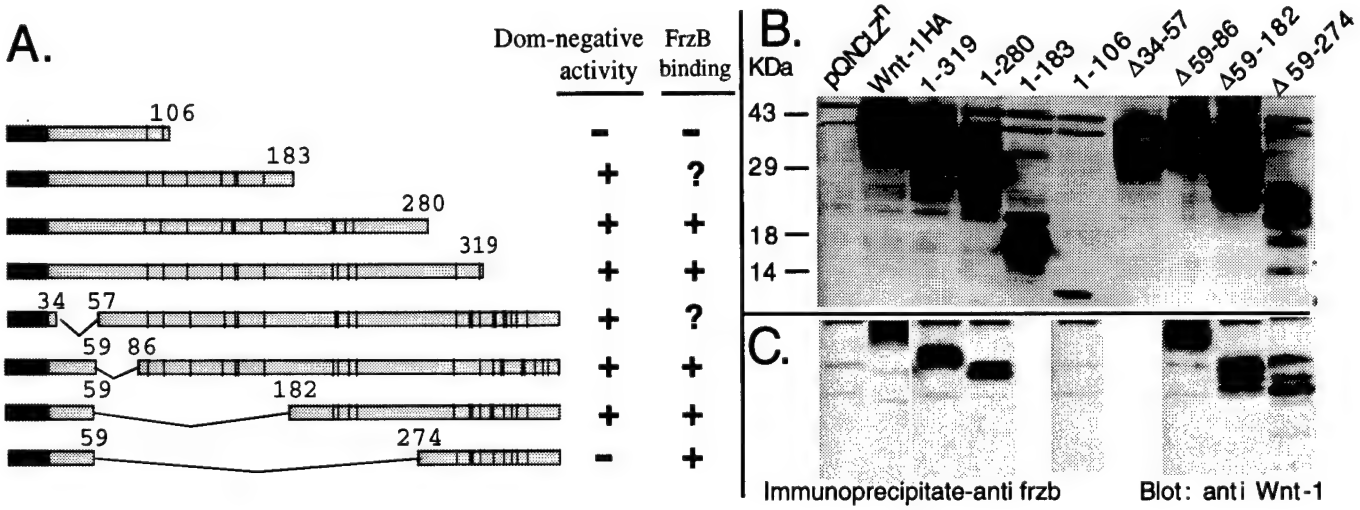


Chapter IV-Figure 1. FrzB antagonizes Wnt-1 signaling in 293 cells.
A. Wnt-1 expression in 293 cells regulates cytosolic β-catenin.
B. Transfection of Frzb expression vector blocks Wnt-1-mediated regulation of cytosolic β-catenin.

We have begun a detailed examination of regions of Wnt-1 that are required for frzb association. A panel of HA-tagged Wnt-1 deletion mutants were prepared and tested for expression in transiently transfected 293 cells (see Figure 2 A,B). Frzb/Wnt-1 interactions were detected within lysates derived from 293 cells co-transfected by frzb and Wnt deletions. Complexes were recovered by anti-frzb antibody and detected by immuno-blotting using anti-HA antibodies (Figure 2C). The data indicate, thus far, at least two discrete binding domains downstream of Wnt-1 codon 182.

In addition, co-expression of Wnts with full length Wnt-1 identified several Wnt deletions that behaved as dominant negative alleles, as judged by their ability to block Wnt regulation of β-catenin. These studies start to define the domains of Wnt-1 proteins required for receptor interaction.

Chapter IV-Figure 2. Expression and Functional analysis of Wnt-1 deletion mutants.
A. schematic diagram of Wnt-1 deletions, Dominant-negative activity and Frzb binding are noted.
B. Immunoblot analysis of Wnt-1 deletions; **C**. Frzb-Wnt-1 deletion co-immunoprecipitations.



V. Analysis of Wnt Signal Transduction and Wnt as a mitogen.

Wnt-1 induces growth, cytosolic β–catenin, and Tcf/Lef transcription in Rat-1 fibroblasts.

Introduction

Wnt gene family encodes secreted factors involved in cell growth, differentiation, organogenesis, and oncogenesis [66]. Wnt proteins have been demonstrated to possess mitogenic [66], inductive [24], and morphogenetic (H. Uyttendaele, personal communication) activities. A mitogenic function of Wnt proteins is supported by the findings that missexpression of *Wnt*-1 promotes mammary tumorigenesis [8,139] or neural tube hyperplasia [140,141]. Wnt-1 expression in mammalian cells results in morphological changes and growth post-confluence in C57MG [68] and RAC311C mammary epithelial cell lines [69], and C3H10T1/2 fibroblasts [84], indicating that Wnt proteins can induce cell growth and loss of contact inhibition. In humans, components of the Wnt signaling cascade have been implicated in development of both colon cancer [79] and melanomas [62].

Understanding of the Wnt signal transduction pathway has come mostly from genetic analyses of the Wnt-1 *Drosophila* orthologue, *wingless (wg)* [12,39]. Wnt/wg signaling events are initiated by receptor activation, that involve the frizzled cell surface protein [42]. This signal suppresses the activity of the Glycogen Synthase Kinase 3 (GSK-3) homologue, zeste-white 3 (zw-3) kinase, leading to changes in phosphorylation and increased stability of the armadillo protein [43]. Armadillo protein is then thought to form complexes with members of *Drosophila Tcf (dTcf)* family transcription factors, regulating expression of *wg* target genes [142].

β–catenin, the mammalian homologue of *Drosophila* armadillo protein, was first identified as a cadherin-associated protein at cell-cell junctions [143]. β–catenin links membrane-bound cadherin and actin-bound a-catenin, coordinating processes essential to cell-cell adhesion and possibly cell migration [55]. In mammalian cells, Wnt-1 expression results in an increase of steady state levels of β–catenin protein, leading to formation of β–catenin/cadherin heterocomplexes [102,144].

β–catenin also associates with the tumor suppressor protein, adenomatous polyposis coli (APC) [56,58]. Wnt-1 expression induces accumulation of β–catenin/APC complexes [101], uncomplexed monomeric β–catenin [101], β–catenin/APC complexes have been identified in human cancer cell lines [62] and are implicated in regulation of steady state levels of β–catenin [144,145]; while monomeric or cytosolic β–catenin are proposed critical to Wnt signaling.

β–catenin is thought to be essential for activating mammalian target genes in response to Wnt signaling [93,146]. Recent studies have provided evidence for interactions between armadillo, or β–catenin, and Tcf/Lef HMG transcription factors in *Drosophila* and *Xenopus* [44,142,147-149]. β–catenin/Tcf transcriptional complexes have been identified in human cancer cell lines and these complexes are implicated in de-regulation of cell growth [79,80]. In addition, a mutant β–catenin displays transforming activity in NIH3T3 cells [77] and transformation of mammary epithelial C57MG cells by Wnts correlates with regulation of β–catenin [124] , further implicating β–catenin as a potential oncogene in mammalian cells [150]. These findings point to a possible role for β–catenin to transduce Wnt-1 proliferative signals in mammalian cells. However, although Wnt-1 regulates β–catenin protein levels, it is not clear if this induction is required or sufficient for Wnt signaling. In addition, it is not known if Wnt-1 regulates Tcf/Lef transcriptional activity, and if this activation is required or sufficient to transduce Wnt-1 proliferative signals in mammalian cells.

We report that Rat-1 fibroblasts show proliferative responses and morphological alterations in response to Wnt-1. Wnt-1 expression results in constitutive up-regulation of cytosolic β–catenin and activation of Tcf/Lef-dependent transcription in Rat-1 cells. However, our data suggest that Tcf/Lef transcriptional activation alone is not sufficient to elicit Wnt-1 proliferative responses in Rat-1 fibroblasts.

Results

Wnt-1 overexpression alters Rat-1 fibroblast morphology and growth.

We set out to identify Wnt-responsive cultured mammalian cell lines. We expressed Wnt-1 in several established human and rat cell lines using a recombinant adenoviral expression vector (Ad) containing an HA-epitope tagged murine *Wnt-1* cDNA (Ad/Wnt-1) (data not shown). Rat-1 fibroblasts [151] responded to Wnt-1 by undergoing changes in morphology and growth. We subsequently characterized the biological and biochemical responses to Wnt-1 using these cells.

Ectopic expression of *Wnt* genes was accomplished with two different gene expression systems: adenoviral and retroviral expression vectors. The adenovirus vector Ad/Wnt-1 was used to infect Rat-1 cells. Wnt-1 proteins were detected by Western blot analysis of cell lysates using anti-HA antibody (12CA5). Twenty-four hours after Ad-infection, Wnt-1 proteins were detected in cells infected by Ad/Wnt-1 (Fig 1A, lane 2), but not found in mock-infected cells (lane 1) or cells infected by a virus expressing the LacZ gene (Ad/LacZ) (lane 7). Wnt-1 expression persisted up to five days after Ad-infection (lanes 2-6).

Stable Rat-1 cell lines expressing *Wnt* genes were established using retroviral vectors driving the expression of HA-epitope tagged *Wnt* cDNAs. We generated *Wnt-1* expressing cell lines and cell lines expressing vector alone (control) or murine *Wnt-5A* (Rat-1/Wnt-5A). Previous studies of Wnt functions in *Xenopus* and mammary epithelial C57MG cells suggest that Wnt-1 and Wnt-5A belong to different functional classes amongst the Wnt protein family members [124](44, 46, 52). We compared the activities of Wnt-1 and Wnt-5A in Rat-1 cells. Western blot analyses of cell lysates revealed that stable Rat-1/Wnt-1 and Rat-1/Wnt-5A cells express glycosylated isoforms of the exogenously expressed Wnt proteins, ranging in molecular mass of 36-45 kDa (Fig 1B). Rat-1/Wnt-1 (Fig. 1B, lane 2) and Rat-1/Wnt-5A (lane 3) cells both showed expression of their respective exogenous Wnt proteins, while control cells showed no exogenous protein expression (lane 1).

Rat-1/Wnt-1 stable cell lines exhibit morphological changes. A similar response to Wnt-1 expression was observed in Rat-1 fibroblasts infected by Ad/Wnt-1 (data not shown). Figure 1C shows that Rat-1/Wnt-1 cells adopt an elongated and refractile appearance, compared to control Rat-1/Wnt-5A cells. Wnt-1 cells grew more densely as a monolayer, forming chord-like bundles lined up in a uniform direction. Rat-1/Wnt-5A exhibited morphologies and growth characteristics identical to uninfected Rat-1 cells, retaining a cuboidal morphology. In subsequent analyses, we will refer to Rat-1/Wnt-5A as control cell lines, as we observed no detectable biological change in response to Wnt-5A expression.

Wnt-1 induces cell growth post-confluence.

One of the growth properties observed in Rat-1 fibroblasts is that they require both contact inhibition and depletion of serum to become growth arrested. To examine whether Wnt-1 can overcome these growth inhibitory signals in Rat-1 cells, we analyzed the ability of Wnt-1 to induce post-confluent growth. Growth post confluence was analyzed in serum free conditions and measured by counting viable cells. Rat-1/Wnt-1 cells continued to proliferate post-confluence in the absence of serum, while control Rat-1/Wnt-5A cells remained growth arrested (Fig 2A). At nine days post-confluence, Rat-1/Wnt-1 cells continued to proliferate in serum-free conditions. This response to Wnt-1 expression was reflected by a three fold increase in relative cell number over that achieved by control Rat-1/Wnt-5A cells (Fig. 2A).

Proliferating Rat-1/Wnt-1 cells packed together tightly, forming dense clusters of cells in a monolayer. These clusters were evident after Giemsa staining of the cell monolayer (Fig 2B). Under similar conditions, Rat-1/Wnt-5A remained growth arrested at confluence, evident by uniform Giemsa staining of the cell monolayer (Fig 2B).

Wnt-1 induces serum-independent cell proliferation.

To test whether Wnt-1 is sufficient to support cell growth in place of mitogenic serum components, we analyzed the growth of Rat-1 stable cell lines in serum-free conditions. Wild-type Rat-1, Rat-1/Wnt-5A, or Rat-1/Wnt-1 fibroblasts were seeded sparsely (<15% confluence) in serum-free media and cell proliferation was measured by counting viable cells at multiple days after serum removal. Wild-type Rat-1 cells remained viable and grew at a very slow rate, which tapered off after 17 days in serum-free culture (Fig 3A). Overexpression of Wnt-1 dramatically increased the growth of Rat-1 fibroblasts in serum-free media (Fig 3A). Rat-1/Wnt-1 cells showed a 2.5 fold increase in cell number, compared to control cells. Control Wnt-5A expressing cells did not achieve confluence after three weeks in culture (Fig 3B), whereas Rat-1/Wnt-1 cells achieved confluence after one week in culture and continued to proliferate post-confluence (Fig 3B). Rat-1/Wnt-1 cells showed a nine-fold total increase in cell number after twenty-four days in culture.

Wnt-1 induces cytosolic accumulation of β–catenin protein in Rat-1 fibroblasts.

It has previously been reported that Wnt-1 expression results in increased steady-state level of β–catenin [101,102,124]. It has also been hypothesized that the accumulation of β–catenin in the cytosol is responsible for Wnt-1 downstream signaling [150]. Having established that Wnt-1 affects the growth and

morphological properties of Rat-1 fibroblasts, we investigated the effects of Wnt-1 on β–catenin protein levels in Rat-1 cells. To study changes in β–catenin in response to Wnt-1, we evaluated β–catenin protein levels in total lysates, and in cytosolic and membranous fractions of Rat-1 cell lysates. Western blot analyses of protein fractions revealed that Wnt-1 expressing cells showed a dramatic increase in cytosolic β–catenin levels compared to control cells (Fig 4B, lane 2). Wild-type Rat-1 and control Rat-1/Wnt-5A cells showed almost undetectable levels of cytosolic β–catenin protein (Fig 4B, lanes 1, 3). β–catenin levels remained constant in total lysates (Fig 4A) and membranous fractions (Fig 4C, lanes 1-3) in all cell lines. Immunofluorescence analyses of Rat-1 cell lines showed a predominant membranous signal, and did not show alterations in cellular localization of β–catenin in response to Wnt-1 expression (data not shown).

Studies in *Xenopus* suggest that Wnt-5A can interfere with Wnt-1 signaling [108]. To study this interaction in Rat-1 cells, we introduced Wnt-1 into Rat-1/Wnt-5A cells by Ad/Wnt-1 infection and measured changes in cytosolic β–catenin levels. Both wild-type Rat-1 cells and Rat-1/Wnt-5A cells showed equivalent responsiveness to Wnt-1, resulting in an acute induction of β–catenin protein in the cytosol at two days after Ad/Wnt-1 infection (Fig 4B, lane 4 and 6). Levels of β–catenin induction by Ad/Wnt-1 in either control (Fig 4B, lane 4) or Rat-1/Wnt-5A cells (lane 6) were comparable to those observed in Rat-1/Wnt-1 stable cell lines (lane 2). These results indicate that Rat-1/Wnt-5A cell lines have not acquired altered responsiveness to Wnt-1 subsequent to selection, and Wnt-5A expression does not interfere with Wnt-1 activity. Ad/Wnt-1 infection of Rat-1/Wnt-1 cells did not further increase cytosolic β–catenin levels (Fig 4B, lane 5). Control Ad/LacZ infection had no effects on β–catenin levels in all three cell lines (Fig 4B, lanes 7-9).

To further examine the potential interaction between Wnt-5A and Wnt-1, we generated an adenoviral expression construct expressing Wnt-5AHA, Ad/Wnt-5A. Infection of Rat-1 cells with Ad/Wnt-5A did not lead to detectable morphological or proliferative changes to the cells (data not shown). Western blot analysis of cell lysates two days following infection showed Wnt-5A proteins were detected in cells infected with Ad/Wnt-5A (Fig 5A, lanes 2-4). Wnt-1 proteins were detected in cells infected with Ad/Wnt-1 (lane 6). No Wnt proteins were detected in mock-infected cells (lane 1) or cells infected with control adenovirus Ad/LacZ (lane 5). By increasing multiplicity of infection (MOI) of Ad/Wnt-5A, a step-wise modest increase in expression of Wnt-5A proteins was detected in Rat-1 cells.

To study the potential modulating effects of Wnt-5A on Wnt-1 activity, we introduced Wnt-5A and Wnt-1 into Rat-1 cells by co-infection with Ad/Wnt-5A and Ad/Wnt-1 and examined the β–catenin levels in cytosolic fractions of cell lysates two days following co-infection. Rat-1 cells showed a substantially increased level of cytosolic β–catenin following infection with Ad/Wnt-1 (Fig. 5B, lane 2; and refer to Fig. 4B). Cells infected with Ad/Wnt-5A showed an almost undetectable level of cytosolic β–catenin (Fig. 5B, lane 4), as did mock-infected cells and cell infected with control Ad/LacZ (lanes 1 and 3). Co-infection of Rat-1 cells with Ad/Wnt-1 and with Ad/Wnt-5A at increasing MOI's of 5, 10 and 20 resulted in no change in the Wnt-1 induction of cytosolic β–catenin level (lanes 5-7). Co-infection with Ad/Wnt-1 and Ad/LacZ also had no effect on Wnt-1 induction of β–catenin. These results suggest that co-expression of Wnt-5A using adenovirus vectors does not interfere with Wnt-1 induction of β–catenin in Rat-1 fibroblasts.

Wnt-1 induces Tcf/Lef transcriptional activation in Rat-1 fibroblasts.

Current models suggest that cytosolic β–catenin interacts with downstream effectors, translocates into the nucleus and activates target genes. One class of potential effectors are the Tcf/Lef HMG transcription factors [98,150]. We postulate that Wnt-1 may directly affect the activities of Tcf/Lef transcription factors by activation of the *Wnt* signal transduction pathway in Rat-1 fibroblasts. To address this hypothesis, we analyzed the effects of Wnt-1 on Tcf/Lef transcriptional activities in Rat-1 fibroblasts, using a Tcf luciferase reporter assay [80].

The Tcf luciferase reporter construct used in these experiments, pTOPFLASH, contains three optimal Tcf binding elements placed in tandem, upstream of a minimal c-*fos* promoter that drives the expression of the luciferase gene. Control Tcf reporter, pFOPFLASH, contains critical nucleotide replacements within the binding elements that disrupt Tcf binding. We observed that co-transfection of *Wnt*-1 expression vector and pTOPFLASH Tcf reporter into Rat-1 fibroblasts resulted in strong luciferase activities two days post-transfection (Fig 6A). Background luciferase activities were measured in cells co-transfected with pTOPFLASH and a LacZ containing plasmid (Fig 6A). Wnt-1 induced three-fold more activation of pTOPFLASH reporter over control pFOPFLASH reporter. Transfections with

increasing amounts of Wnt-1 plasmids (0.5, 1, or 5 mg) did not result in increasing activation of pTOPFLASH reporter (Fig 6A).

Next, we investigated if there are constitutive Tcf/Lef transcriptional activities in the stable Rat-1 cell lines. Rat-1/Wnt-1 and Rat-1/Wnt-5A cells were transfected with either pTOPFLASH or control pFOPFLASH reporters, and luciferase activities were measured two days later. Figure 6B illustrates that Rat-1/Wnt-1 cells showed three-fold greater constitutive Tcf-dependent luciferase activities than control Rat-1/Wnt-5A cells. Rat-1/Wnt-1 and Rat-1/Wnt-5A showed comparable background luciferase activities when transfected with the mutant Tcf reporter, pFOPFLASH (Fig 6B). Control transfections using a constitutive reporter (pSV2-Luc) that uses the SV40 early promoter to express luciferase, demonstrated that transfection efficiencies were comparable in all stable cell lines (data not shown).

Mutant β–catenin (S37A) does not accumulate in the cytosol.

It has been postulated that cytosolic accumulation of β–catenin is the key event that drives the formation of active β–catenin/Tcf transcriptional complexes. Cytosolic β–catenin accumulation would thus be the rate-limiting step in Wnt-1 signaling. Wild-type cytosolic β–catenin is generally unstable and degrades rapidly in the absence of Wnt-1 signal [152]. A mutant β–catenin with a serine to alanine mutation at residue 37 (S37A) is thought to be more stable, and thus more active than wild-type β–catenin [62].

To examine the activities of β–catenin, we expressed β–cateninS37A in Rat-1 fibroblasts. Using a recombinant retrovirus expressing HA-epitope tagged β–cateninS37A, we generated a polyclonal Rat-1 stable cell line, Rat-1/β–CatS37A. Expression of β–cateninS37A protein in Rat-1/β–CatS37A cells was shown by Western blot analyses of total cell lysates using anti-HA antibodies. Figure 7A (lane 2) shows the HA-tagged β–cateninS37A protein in total lysates of Rat-1/β–CatS37A cells, migrating at approximately 92kD. No exogenous protein was detected in mock-infected cells (Fig 7A, lane 1). In parallel, Western blot analysis using anti-β–catenin antibodies showed exogenous β–cateninS37A proteins (Fig 7A, lane 4, indicated by arrow) migrating slightly higher than endogenous β–catenin proteins (Fig 7A, lane 3). The observed alteration in electrophoretic mobility of β–cateninS37A is likely due to the HA-epitope tag.

We next analyzed the levels of β–cateninS37A proteins in the cytosolic fraction of Rat-1/β–CatS37A cells. By cell fractionation and Western blot analysis using anti-β–catenin antibodies, we observed little accumulation of β–cateninS37A protein in the cytosolic fraction (Figure 7B, lane 3), as compared to Wnt-1 expressing cells (Fig 7B, lane 1). Like Rat-1/Wnt-5A cells, Rat-1/β–CatS37A cells showed low levels of cytosolic β–catenin (Fig 7B, lanes 2 and 3). However, both cell lines showed acute responsiveness to Wnt-1, demonstrated by the dramatic accumulation of β–catenin protein in the cytosol two days following Ad/Wnt-1 infection (Fig 7B, lane 5 and 6). Control infection with Ad/LacZ had no effects on β–catenin levels in any of the cell lines (Fig 7B, lanes 7-9). The cytosolic pool of β–catenin in Rat-1/β–CatS37A cells following Ad/Wnt-1 infection is primarily composed of endogenous β–catenin. Ectopically expressed β–cateninS37A, which was detected as a slower migrating form, shows little or no responsiveness to the Wnt-1 signal and does not accumulate in the cytosol following Ad/Wnt-1 infection (data not shown) (see Discussion).

β–cateninS37A does not affect growth of Rat-1 fibroblasts.

We next characterized the growth of Rat-1/β–CatS37A cells in serum-free conditions. When seeded sparsely, Rat-1/β–CatS37A cells proliferated at a rate similar to that of wild-type Rat-1 cells (Fig 8A) and did not achieve confluence in the absence of serum (Fig 8B). Rat-1/β–CatS37A cells showed a rate of proliferation significantly slower than that of Rat-1/Wnt-1 cells (Compare Fig 3A to Fig 8A). Rat-1/β–CatS37A cells retained the morphological and growth properties of wild-type Rat-1 fibroblasts (Fig 8B). In addition, Rat-1/β–CatS37A cells failed to grow post-confluence in serum-free media (data not shown), as seen for Rat-1/Wnt-1 cells. We have also generated and analyzed eight clonal lines derived from the Rat-1/β–CatS37A cell line. Although there are clonal differences in expression levels of β–cateninS37A, as determined by western blotting, there is little observable differences in growth and morphology between the polyclonal and clonal lines of Rat-1/β–CatS37A (data not shown).

β–cateninS37A induces Tcf/Lef transcriptional activation.

β–catenin mutations have been implicated in constitutive Tcf transcriptional activation in cancer cell lines [79]. We examined if ectopic expression of β–cateninS37A leads to activation of Tcf/Lef

dependent transcription in Rat-1 fibroblasts. Rat-1 fibroblasts were co-transfected with Tcf luciferase reporter constructs and β–cateninS37A expression plasmids. Luciferase activities were measured two days after transfection. A dose-dependent increase in luciferase activities was detected by co-transfecting increasing amounts of β–cateninS37A cDNA with pTOPFLASH reporter, reaching up to five fold over activation of pFOPFLASH control reporter (Fig 9A). Control transfections of pTOPFLASH reporter with LacZ plasmids showed background luciferase activities (Fig 9A).

We next investigated whether Rat-1/β–CatS37A stable cell lines exhibited constitutive Tcf/Lef transcriptional activities. We introduced pTOPFLASH or pFOPFLASH reporters into Rat-1 stable cell lines by transient transfection and measured luciferase activities two days after transfection. Rat-1/β–CatS37A cells showed strong constitutive Tcf/Lef transcriptional activities. Figure 9B illustrates that Rat-1/β–CatS37A and Rat-1/Wnt-1 cells showed comparable levels of luciferase activities when transfected with pTOPFLASH Tcf reporter. In both cell lines, no significant luciferase activities were detected in cells transfected with control pFOPFLASH reporter. Control Wnt-5A cells showed no significant luciferase activity when transfected with either Tcf reporter constructs (Fig 9B). We conclude that expression of the mutant form β–cateninS37A activates Tcf/Lef dependent transcription in Rat-1 cells. Stable expression of either Wnt-1 or β–cateninS37A in Rat-1 fibroblasts results in constitutive Tcf/Lef transcriptional activation.

Discussion

In this study, we have identified Rat-1 fibroblasts as a Wnt-1-responsive cell line. Wnt-1 supports growth of Rat-1 cells in the absence of serum and stimulates growth beyond confluence. We have used Rat-1 cells to characterize Wnt signaling events and to relate these events to biological responses of Rat-1 cells to Wnt-1. Our data show that activation of the Wnt signal transduction pathway results in induction of β–catenin in the cytosol and Tcf/Lef transcriptional activation in Rat-1 fibroblasts.

We proposed that the biological phenotype of Rat-1 fibroblasts in response to Wnt-1 is a result of increased levels and/or activities of β–catenin, a key Wnt signaling intermediate. Studies in *Xenopus* have provided evidence that overexpression of β–catenin can mimic Wnt activities [53,153]. Examination of β–catenin protein levels in Rat-1 fibroblasts in response to Wnt-1 revealed an acute accumulation of β–catenin in the cytosolic cellular fraction. In contrast, little or no change in β–catenin levels were observed in the membranous fractions of these cells, indicating a selective modulation of cytosolic β–catenin by Wnt-1. As the membranous pool contains the majority of β–catenin in Rat-1 fibroblasts, the total cellular β–catenin was not noticeably changed by Wnt-1. Previous studies have shown that Wnt-1 stabilizes β–catenin as monomers and complexes with cadherin or APC in mammary epithelial cells [101]. Our studies show Wnt-1 induces a specific accumulation of β–catenin in the cytosol and are consistent with analysis of β–catenin in other established cell lines [127]. We also found that β–catenin levels were not altered in response to Wnt-5A expression, consistent with previous reports of different activities for Wnt-1 and Wnt-5A [88,104,107]. We did not find evidence that Wnt-5A expression could interfere with Wnt-1 signaling in Rat-1 fibroblasts, in contrast to studies of the activity of Wnt-5A in *Xenopus* [108]. It can be speculated that interactions between Wnt-5A and Wnt-1 are different in mammalian tissue culture cell lines than in Xenopus embryos. It is also possible that significant overexpression of Wnt-5A relative to Wnt-1 expression is required in order to exhibit an inhibitory effect on Wnt-1 activity.

One proposed function of cytosolic β–catenin is interaction with Tcf/Lef family transcription factors, activating downstream Wnt target genes. Tcf/Lef functions have been shown to be critical in lymphoid development and inductive epithelial-mesenchymal interactions [154,155]. Recent findings in *Xenopus* and *Drosophila* have suggested that Tcf/Lef transcriptional activation is part of the Wnt/wingless signal transduction pathway [44,142,147,149]. However, there is no evidence of a direct link between Wnt signal and Tcf/Lef transcription in mammalian cells. Addressing this question, we examined Wnt-1 induction of Tcf/Lef transcriptional activities in Rat-1 fibroblasts. In both transient and stable systems, we observed that Wnt-1 expression results in strong activation of Tcf/Lef transcriptional elements. The observed Wnt-1 induction of Tcf/Lef transcriptional activities is specifically correlated with Wnt-1 induction of β–catenin in Rat-1 cells. Our findings in Rat-1 fibroblasts are consistent with current genetic models [98], pointing to a link between Wnt signal, β–catenin induction and Tcf/Lef transcriptional activation.

The biological phenotype of Rat-1 fibroblasts in response to Wnt-1 signal suggests mitogenic activities of Wnt-1. Activities of Wnt-1 in mammalian cells have previously been examined by ectopic expression of Wnt-1 in mammary epithelial cells [68,69] or mouse fibroblasts [84]. Similar to previous

findings, we observed that ectopic expression of Wnt-1 is sufficient to induce growth post-confluence in quiescent Rat-1 fibroblasts. In contrast to previous studies reporting Wnt-1 insufficient to support cell growth in low serum [84], we found that Wnt-1 enhances serum-independent growth of Rat-1 fibroblasts. The mechanism(s) by which Wnt-1 increases Rat-1 cell numbers is these assays is not known. Wnt-1 may act as a mitogen to support cell growth, which is indicated by the fact that Wnt-1 expressing cells showed significant growth post-confluence. However, it may act as a cell survival factor in serum-deprived conditions, hence increasing the number of viable cells. Wnt-5A does not have similiar biological effects on Rat-1 fibroblasts, suggesting that the observed phenotype is due to Wnt-1-specific activities and not to a pan Wnt activity; thus, Wnt-5A serves as an appropriate negative control in our experiments.

The observed growth response to Wnt-1 in Rat-1 fibroblasts are correlated with β–catenin induction and Tcf/Lef transcriptional activation. To explore the role of β–catenin, we generated Rat-1 stable cell lines expressing mutant β–catenin (S37A). Mutation of serine residue 37 of β–catenin (S37A) was originally identified in melanoma cell lines [62,79,80]. S37A may interfere with glycogen synthase kinase-3 beta (GSK-3b) phosphorylation of β–catenin, and may lead to stabilization of cytosolic β–catenin [57]. However, in Rat-1 fibroblasts, we found that mutant β–cateninS37A did not accumulate in the cytosol. One interpretation of this finding is that alternate GSK-3b phosphorylation sites remain active and render rapid degradation of β–cateninS37A. Alternatively, β–cateninS37A may require additional modifications by Wnt-1 signal to accumulate in the cytosol. Interestly, β–cateninS37A is relatively insensitive to Wnt-1 signal, showing little accumulation in the cytosol following Ad/Wnt-1 infection (data no shown). It is possible then that residue serine 37 may be necessary for additional modifications of β–catenin by the Wnt-1 signal to accumulate in the cytosol.

Expression of β–cateninS37A, however, results in strong constitutive Tcf/Lef transcriptional activities in Rat-1 cells, in the absence of Wnt-1 signal. The magnitude of activation seen in β–cateninS37A expressing cells were comparable to those seen in Wnt-1 expressing cells. Introducing increasing amounts of β–catenin expression plasmid resulted in increasing transcriptional activation, while introducing increasing amounts of Wnt-1 did not lead to increasing activation. Our data support that β–catenin is a rate-limiting intermediate in Wnt-1 induction of Tcf/Lef transcriptional activation.

Constitutive Tcf/Lef transcriptional activity has recently been identified in colon cancer cell lines [79,80]. Mutations in β–catenin or mutations in adenomatous polyposis coli (APC) tumor suppressor gene are associated with constitutive Tcf/Lef transcriptional activity by a β–catenin/Tcf complex in these cell lines. Together, these findings implicate that deregulation of β–catenin or APC can lead to constitutive β–catenin/Tcf transcriptional activation, which may contribute directly to tumorigenesis. One interpretation of our findings is that Wnt-1 induces β–catenin and constitutive Tcf/Lef transcriptional activity, resulting in proliferative responses in Rat-1 fibroblasts.

Interestingly, we found that induction of Tcf/Lef transcriptional activity is not sufficient to alter cell growth and morphology in place of Wnt-1. Although Rat-1/Wnt-1 and Rat-1/β–CatS37A cells show comparable magnitudes of Tcf/Lef transcriptional activation, these cell lines do not show comparable biological phenotypes. One interpretation of these data is that S37A mutation in β–catenin renders aberrant transcriptional activity, independent of other potential signaling functions of β–catenin. Western blot analysis of nuclear fractions of Rat-1/β–CatS37A cell lysates showed some localization of β–cateninS37A in the nuclei (data not shown). It is thus possible that β–cateninS37A can translocate into the nucleus and may have increased transcriptional activity. Another interpretation of these data is that Tcf/Lef transcription may be one of many downstream events in the Wnt-1 signal transduction pathway, and that Tcf/Lef transcriptional activation alone is not sufficient to elicit proliferative effects in Rat-1 fibroblasts in place of Wnt-1. However, we also recognize that the Tcf/Lef transcriptional activity measured by reporter constructs [80] may be saturable and may not represent endogenous gene activation.

Cytosolic accumulation of β–catenin in response to Wnt-1 may have additional growth signaling functions aside from Tcf/Lef transcriptional activation. β–cateninS37A is functional in transcriptional activation but does not accumulate in the cytosol or enhance growth. We propose that accumulation of cytosolic β–catenin may not strictly function to activate Tcf-dependent transcription but may also be required to transmit Wnt-1 growth signals. Modest changes in β–catenin levels may be sufficient to form functional β–catenin/Tcf complex, activating transcription. However, accumulation of a larger pool of cytosolic β–catenin may be necessary for Wnt-1 growth signals, for instance, by interactions with other cytosolic proteins. Considering β–catenin functions in cell adhesion, it is also possible that Wnt-1 expression in Rat-1 cells modulates β–catenin functions in cell adhesion, as reported in other settings

[85,102,156]. Consequently, changes in adhesive properties may alter cellular morphology and growth, eliciting Wnt-1 growth effects. In an alternative model, the Wnt-1 signal transduction pathway may bifurcate at a point upstream of β–catenin. As a result, a secondary signal upstream of β–catenin may contribute to Wnt-1 proliferative effects in Rat-1 fibroblasts. Experiments are now in progress to further address potential signaling events critical to Wnt-1 growth signals.

Materials and methods

Adenoviral expression vectors.
Recombinant adenovirus vectors expressing either Wnt-1 or Wnt-5A from the cytomegalovirus immediate early promoter were constructed by cre/lox recombination [157]. To accomplish this, Wnt-1HA and Wnt-5AHA cDNA's [124] were subcloned into the pAdlox shuttle vector creating pAdlox Wnt-1HA and pAdlox Wnt-5A, which were then recombined with donor virus to create Ad/Wnt-1 and Ad/Wnt-5A. These adenovirus vectors contain the Wnt-HA expression cassette replacing the E1 region genes and a 2.6 kb deletion in the E3 region [158]
Retroviral expression vectors.
Mammalian retroviral expression vectors, pZNCX and pQNCX, were used to construct and express full-length murine *Wnt-1*, murine *Wnt-5A*, human *β–catenin (S37A)*. cDNA expression in pZNCX and pQNCX is driven by an internal cytomegalovirus (CMV) enhancer/promoter (unpublished observations). cDNA for *Wnt-5A* was generously provided by Andrew McMahon (Harvard University). cDNA for mutant *β–catenin* (S37A) was generously provided by Steven Byers (Georgetown University). All cDNAs are modified to encode a hemagglutinin (HA) epitope tag on the 3' end.
Cell culture.
Rat-1 fibroblasts [151] and BOSC 23 packaging cells [111] were maintained in Dulbecco's modified Eagle (DMEM) medium, supplemented with 10% fetal bovine serum and penicillin-streptomycin, at 37C, 8% CO2 and 90% humidity. To measure growth beyond confluence, Rat-1 cells were seeded at approximately 75% confluence and allowed to achieve confluence in the presence of serum overnight. The following day, serum was removed by rinsing cell monolayers once with phosphate-saline buffer (PBS) and once with DMEM. Cells were then cultured in DMEM. At several time points after serum removal, cells were dissociated by trypsinization and viable cells were counted after Trypan blue staining. To visualize cellular growth post-confluence, cell monolayers were fixed with cold methanol:acetone (1:1), stained with Giemsa nuclear stain, and photographed. To measure serum-independent cell proliferation, Rat-1 cells were seeded at approximately 15% confluence in the presence of serum. The following day, serum was removed and at several time points after serum removal, viable cell numbers were obtained as described above.
Adenoviral infection.
Rat-1 fibroblasts were seeded at 80% confluence and were subsequently mock infected or infected with recombinant adenoviruses at various multiplicity's of infection (MOI's). Prior to infection, cells were rinsed with DMEM. Infections were carried out using virus-containing inoculum, in DMEM containing 2% fetal bovine serum, added to cells for one hour at 37C. Cells were then maintained in DMEM containing 2% fetal calf serum.
Transient transfection and retroviral infection.
Recombinant retroviruses were generated by calcium-phosphate tranfecting the retroviral constructs into BOSC packaging cells [111]. One day later, BOSC cells were treated with mitomycin C (10mg/ml) (Sigma) for four hours. Twenty-four hours after treatment, transfected BOSC cells were trypsinized and seeded with target Rat-1 fibroblasts for co-culture. After forty-eight hours, cells were cultured with media containing 500mg/ml Geneticin (G418) (Gibco). Resistant colonies were pooled as polyclonal, stable cell lines, maintained in medium containing 250mg/ml Geneticin.
Western blot analysis.
Wnt-1, Wnt-5A and β–cateninS37A protein expression was assessed by Western blot analysis of cell lysates. Briefly, cells were washed twice with cold PBS, pelleted, and then resuspended in lysis buffer (50mM Tris (pH8); 150 mM NaCl; 1% TritonX100; 10mg/ml Aprotonin; 0.5mM phenylmethylsulfonyl fluoride (PMSF); 2mg/ml Leupeptin; 2mg/ml Pepstatin) for 30 mins on ice. Cell lysates were then clarified by microcentrifugation at 10,000 rpm for 10 mins at 4C. Protein concentrations were determined using BioRad Protein determination dye. Fifty micrograms cellular proteins were separated in 10% SDS-polyacrylamide (PAGE) gels and transferred onto nitrocellulose filters.

Filters were blocked with TBST (10mM Tris (pH8); 150mM NaCl; 0.2% Tween-20) containing 1% bovine serum albumin (BSA) (fraction V), and immunoblotted for protein expression by incubating with anti-HA monoclonal antibody 12CA5 (Berkeley Antibody Co.) at 1:50 dilution in TBST/1% BSA for 2 hours at room temperature. Blots were washed for 25 mins with TBST at room temperature and were exposed to secondary antibody, horseradish peroxidase-conjugated sheep anti-mouse immunoglobullin G (Amersham) at 1:5000 dilution for 45 mins at room temperature, followed by a TBST wash at room temperature. Protein expression was visualized by enhanced chemiluminescence detection reagents (Amersham) and exposure to X-ray film.

Cell fractionation and β–catenin analysis.

β–catenin protein levels were assessed in cytosolic and membranous pools prepared by fractionation of cellular lysates as described [124](Giarre, M., Dong, Q., and Brown, A.M.C, in preparation). Briefly, cells were washed twice with cold PBS and collected in physiological buffer (PB) (10mM Tris (pH 7.4); 140mM NaCl; 5mM EDTA; 2mM DTT; 1mM PMSF; 1mg/ml aprotonin; 2mg/ml leupeptin; 2mg/ml pepstatin) (500ml per 100mm confluent dish). Cells were homogenized in PB by 30 strokes of a dounce homogenizer and the lysate centrifuged for 10 mins at 3,000 rpm using a microcentrifuge, or 500 x g at 4C. Membranous and cytosolic material was obtained by ultracentrifugation at 30,000 rpm or 100,000 x g for 90 mins at 4C. The supernatant was designated the cytosolic fraction, and pellets were resuspended in physiological buffer containing 0.1% SDS (150ml per 100mm confluent dish) and designated the membranous fraction. Protein concentrations of respective fractions were determined by BioRad Protein determination dye. β–catenin was analyzed by separation of 30 mg cytosolic proteins or 15 mg membranous proteins on 10% SDS-polyacrylamide gels, and Western blot analyses using an anti-β–catenin monoclonal antibody (Transduction Laboratories), at 1:5000 dilution in TBST with 1% BSA for two hours at room temp. Protein expression was visualized by enhanced chemiluminescence detection reagents (Amersham) and exposure to X-ray film.

Tcf luciferase assay.

pTOPFLASH and pFOPFLASH Tcf luciferase reporter constructs were generously provided by Hans Clevers (University Hospital, Utrecht, The Netherlands) [80]. pSV-Luc luciferase reporter construct utilizing the SV40 early promoter to drive constitutively the expression of luciferase gene, was generously provided by Andrew Henderson, Penn State University, PA. Four micrograms of reporter construct was introduced into cells by calcium phosphate transfection method [111]. Two days after transfection, cells were collected and resuspended in lysis buffer containing 5% Triton; 125mM Gly-Gly (pH 7.8); 75mM MgSO4; 20mM EGTA (pH7.8); 10mM DTT. Crude lysates were clarified by pulse microcentrifugation. Supernatants were evaluated for luciferase activities using luciferin substrates (Sigma). Measurements were obtained from transfections performed in triplicate and averaged (n=3).

Figure Legends for chapter V.

FIG. 1. Wnt-1 expression alters Rat-1 fibroblast morphology and growth.

(A) Transient expression of Wnt-1 protein by recombinant adenoviral expression vector in Rat-1 fibroblasts. Western blot analysis of cell lysates shows Wnt-1 protein expressed in cells at multiple days (1-5) post infection with Ad/Wnt-1 (lane 2-6) (MOI=5), but not with control Ad/LacZ (Z) (MOI=5) (lane 7) or mock infection (M) (lane 1). Wnt-1 was detected by anti-HA antibody (Anti-HA).

(B) Stable expression of Wnt-1 and Wnt-5A proteins in Rat-1 stable cell lines, Rat-1/Wnt-1 and Rat-1/Wnt-5A. Western blot analysis of cell lysates shows Wnt-1 (lane 2) and Wnt-5A (lane 3) proteins expressed, as compared to control cells (lane 1). Wnt-1 and Wnt-5A proteins were detected by anti-HA antibody.

(C) Wnt-1 alters Rat-1 fibroblasts morphology and growth. Phase contrast microscopy of polyclonal Rat-1/Wnt-1 and Rat-1/Wnt-5A stable cell lines. Rat-1/Wnt-1 cells attain a more elongated, refractile morphology and densely pack in chord-like bundles, as compared to Rat-1/Wnt-5A cells.

FIG. 2. Wnt-1 induces proliferation in quiescent Rat-1 fibroblasts.

Rat-1/Wnt-1 (–✱–) and control Rat-1/Wnt-5A (–◇–) cells were cultured to confluence, deprived of serum, and monitored for growth post-confluence.

(A) At multiple days post-confluence, viable cells were trypsinized and counted using trypan blue exclusion method. Percent relative cell number is calculated as percentage relative to cell number recorded at one day after serum removal. Triplicate samples were counted and averaged (n=3).

(B) Six days post-confluence, cell monolayers were fixed and stained with Giemsa nuclear stain and photographed. Scale bar represents 4.75 mm.

FIG. 3. Wnt-1 induces serum-independent growth in Rat-1 fibroblasts.
Wild-type Rat-1 (—□—) , Rat-1/Wnt-1 (—✱—) and control Rat-1/Wnt-5A (—◇—) cells were seeded at low density (<15% confluence), deprived of serum, and monitored for serum-independent growth.
(A) At multiple days after removal of serum, cells were trypsinized and viable cells were counted. % Relative cell number is calculated as percentage relative to cell number recorded at one day after serum removal. Triplicate samples were counted and averaged (n=3).
(B) At multiple days (Days 1, 8, 17) after removal of serum, Rat-1/Wnt-1 and Rat-1/Wnt-5A cells were photographed and monitored for serum-independent growth.

FIG. 4. Wnt-1 induces cytosolic accumulation of β–catenin protein in Rat-1 fibroblasts.
Western blot analyses of steady-state β–catenin protein levels in (A) unfractionated, (B) cytosolic and (C) membranous protein fractions of wild-type Rat-1 (blank), Rat-1/Wnt-1 (W1) and Rat-1/Wnt-5A (W5A) cells. Cells were uninfected or infected with Ad/Wnt-1 (Ad/Wnt-1) or with control Ad/LacZ (Ad/LacZ) (MOI=5). Cell extracts were prepared and fractionated, as described in Materials and methods. Protein fractions were separated on 10% SDS-PAGE, transferred onto nitrocellulose, and analysed by Western blot analysis using anti-β–catenin antibody (Anti-β–Cat).

FIG. 5. Co-expression of Wnt-5A does not interfere Wnt-1 induction of cytosolic β–catenin in Rat-1 fibroblasts.
(A) Expression of Wnt-5A protein in Rat-1 cells at increasing MOI with Ad/Wnt-5A. Western blot analysis of cell lysates at two days following infection with Ad/Wnt-5A at an MOI of 5, 10 and 20, or with Ad/Wnt-1 (W1) (MOI=5), Ad/LacZ (Z) (MOI=5) or mock infection (M). Wnt-1 and Wnt-5A proteins are detected by anti-HA antibody (Anti-HA).
(B) Co-expression of Wnt-5A does not interfere with Wnt-1 induction of cytosolic β–catenin in Rat-1 fibroblasts. Western blot analyses of steady-state cytosolic β–catenin protein levels in Rat-1 cells following mock infection (M), infection with Ad/Wnt-1 (W1), Ad/LacZ (Z), Ad/Wnt-5A (5A), co-infection with Ad/Wnt-1 (MOI=5) and Ad/Wnt-5A (MOI's=5, 10, 20), or co-infection with Ad/Wnt-1 (W1) (MOI=5) and Ad/LacZ (Z) (MOI=20). Cell extracts were prepared, fractionated, and analyzed by Western blot analysis using anti-β–catenin antibody (Anti-β–Cat).

FIG. 6. Wnt-1 induces Tcf/Lef-dependent transcription in Rat-1 fibroblasts.
(A) Transient expression of Wnt-1 induces Tcf/Lef-dependent transcription. Wild-type (pTOPFLASH; □) or mutant (pFOPFLASH; ■) Tcf reporter constructs (4mg) were co-transfected into Rat-1 fibroblasts with increasing amounts (mg) of Wnt-1 or LacZ cDNA.
(B) Stable expression of Wnt-1 in Rat-1/Wnt-1 cells induces constitutive Tcf/Lef transcriptional activation. Wild-type Tcf (pTOP) or mutant Tcf (pFOP) reporter constructs (4mg) were transfected into Rat-1/Wnt-1 (■) or Rat-1/Wnt-5A (□) stable cell lines. Luciferase activities were measured two days after transfection. Triplicate samples were counted and averaged (n=3).

FIG. 7. Mutant β–catenin (S37A) does not accumulate in the cytosol.
(A) Western blot analyses of β–CatS37A in Rat-1/β–CatS37A stable cell lines. Cell lysates were separated on 10% (lanes 1 and 2) or 7.5% (lanes 3 and 4) SDS-PAGE, transferred onto nitrocellulose, and β–CatS37A was detected by Western blot analysis using anti-HA antibody (lane 2) or anti-β–catenin (lane 4). Lane 1 shows no exogenous protein is detected in control cells. Lane 3 shows endogenous expression of β–catenin in Rat-1 cells.
(B) Western blot analysis of cytosolic β–catenin protein levels in Rat-1/Wnt-1 (W1), Rat-1/Wnt-5A (W5A) and Rat-1/β–CatS37A(S37A) stable cell lines. Cells were uninfected or infected with Ad/Wnt-1 (Ad/Wnt-1) (MOI=5) or with control Ad/LacZ (Ad/LacZ) (MOI=5) Cytosolic protein fractions were separated on 10% SDS-PAGE, transferred onto nitrocellulose, and analyzed by Western blot analysis using anti- β–catenin antibody (Anti-β–Cat).

FIG. 8. Mutant β–catenin (S37A) expression does not alter Rat-1 morphology or growth properties. Rat-1/β–CatS37A (—◇—) and wild-type Rat-1 (—□—) cells were seeded at low density (<15% confluence), deprived of serum, and monitored for serum-independent growth.
(A) At multiple days after removal of serum, cells were trypsinized and viable cells were counted. Percent relative cell number is calculated as percentage relative to cell number recorded at one day after serum removal. Samples were counted in triplicate.
(B) After removal of serum, wild-type Rat-1 and Rat-1/β–CatS37A cells were photographed on days as indicated in the figure.

FIG. 9. Mutant β–catenin (S37A) activates Tcf/Lef-dependent transcription.
(A) Transient expression of β–CatS37A induces Tcf/Lef-dependent transcription. Wild-type (pTOPFLASH; □) or mutant (pFOPFLASH; ■) Tcf reporter constructs (4mg) were co-transfected into Rat-1 fibroblasts with increasing amounts (mg) of β–CatS37A or control LacZ cDNA's.
(B) Stable expression of β–CatS37A in Rat-1/β–CatS37A cells induces constitutive Tcf/Lef transcriptional activation. Wild-type Tcf (pTOP) or mutant Tcf (pFOP) reporter constructs (4mg) were transfected into Rat-1/β–CatS37A (▨), Rat-1/Wnt-1 (■) or Rat-1/Wnt-5A (□) stable cell lines. Luciferase activities were measured two days after transfection. Triplicate samples were counted and averaged (n=3).

Chapter V

Figure 1

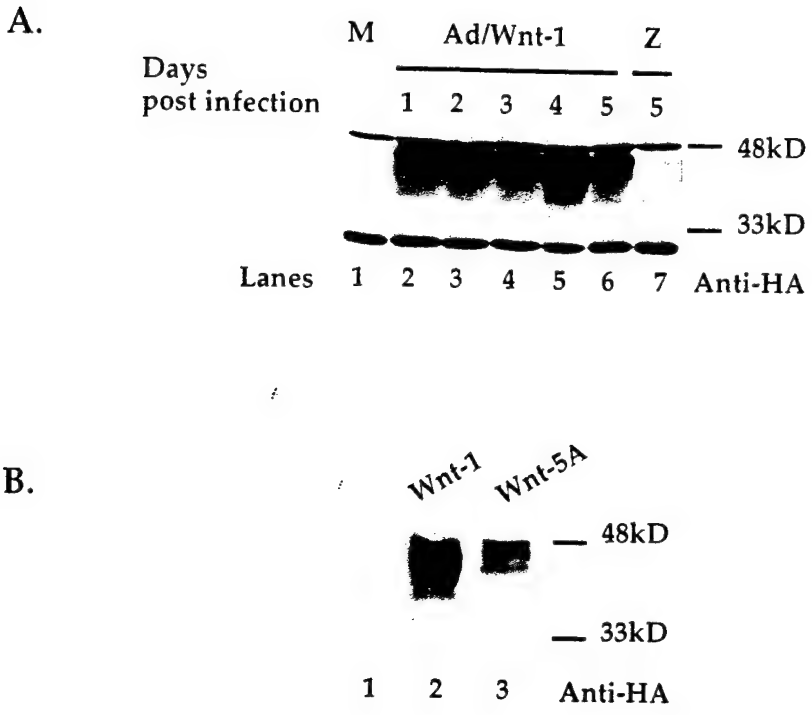


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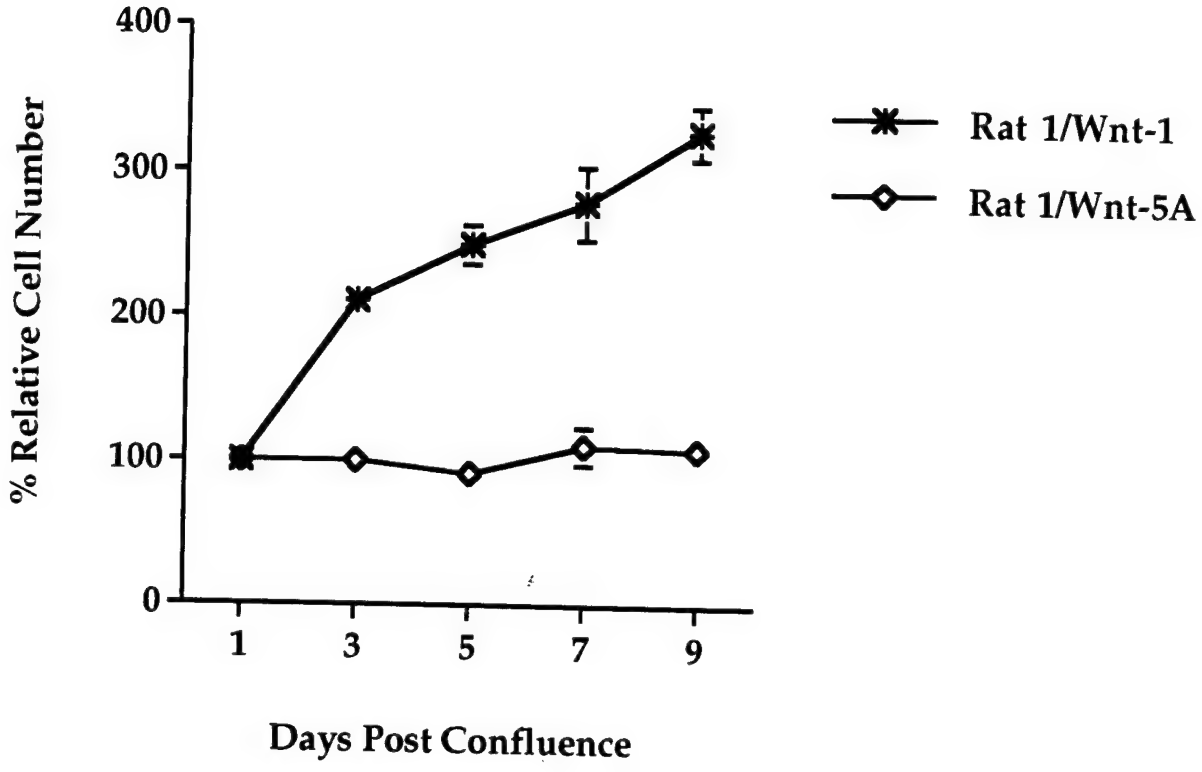
Figure 2

A.



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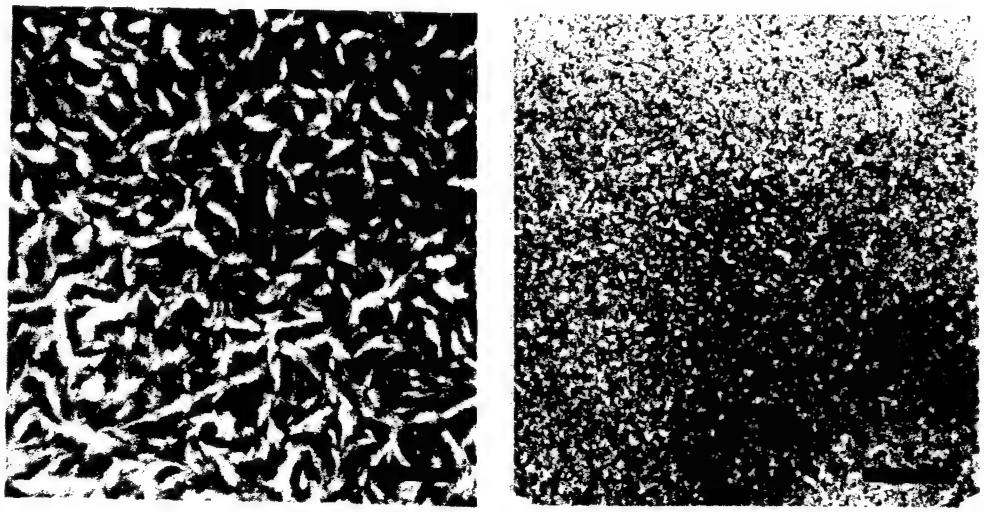

Rat 1/Wnt-1 Rat 1/Wnt-5A

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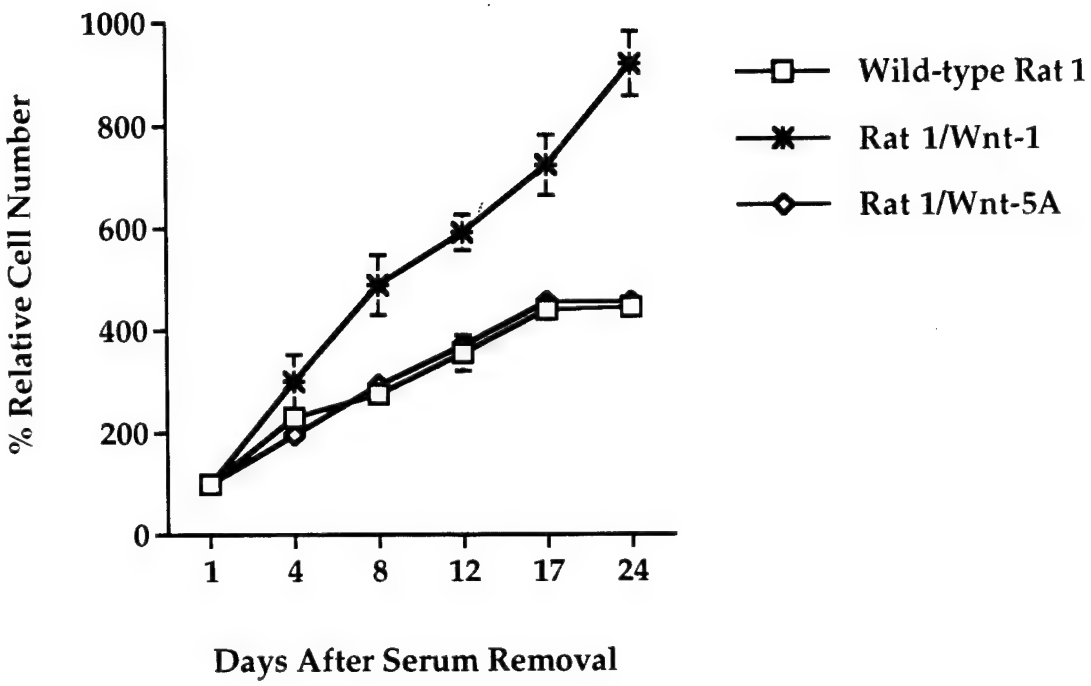
Figure 3

A.



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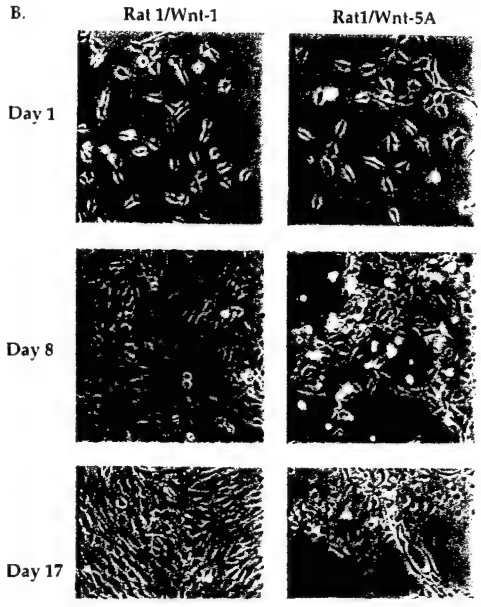
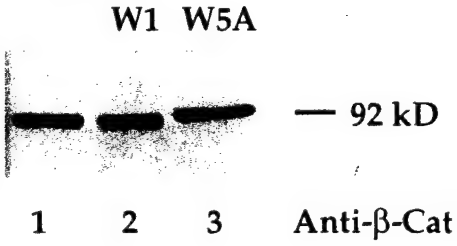


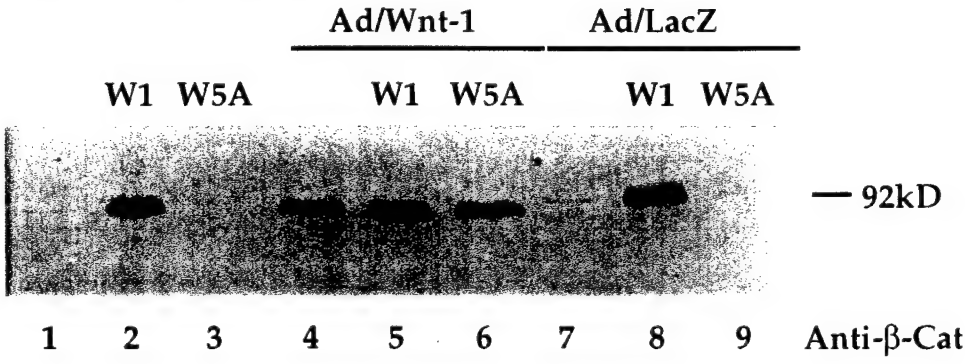
Chapter V

Figure 4

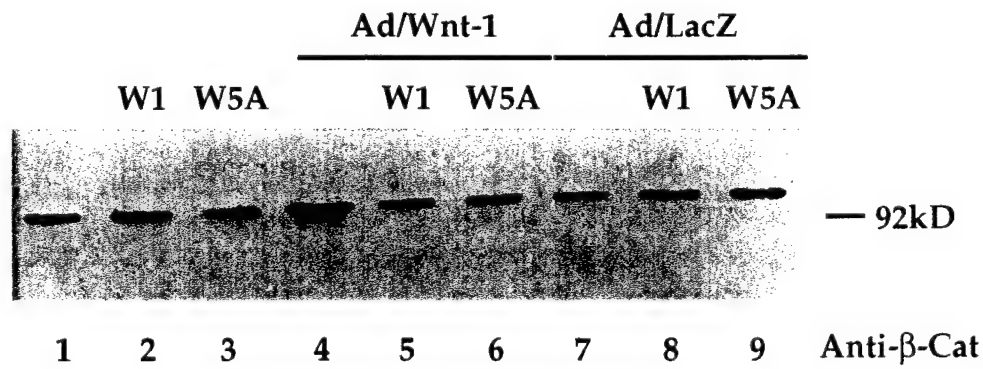
A. Total Lysates



B. Cytosolic Fractions



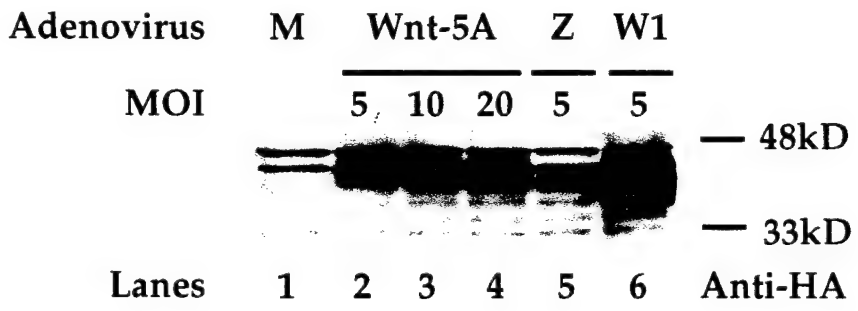
C. Membranous Fractions



Chapter V

Figure 5

A. Total Lysates



B. Cytosolic Fractions

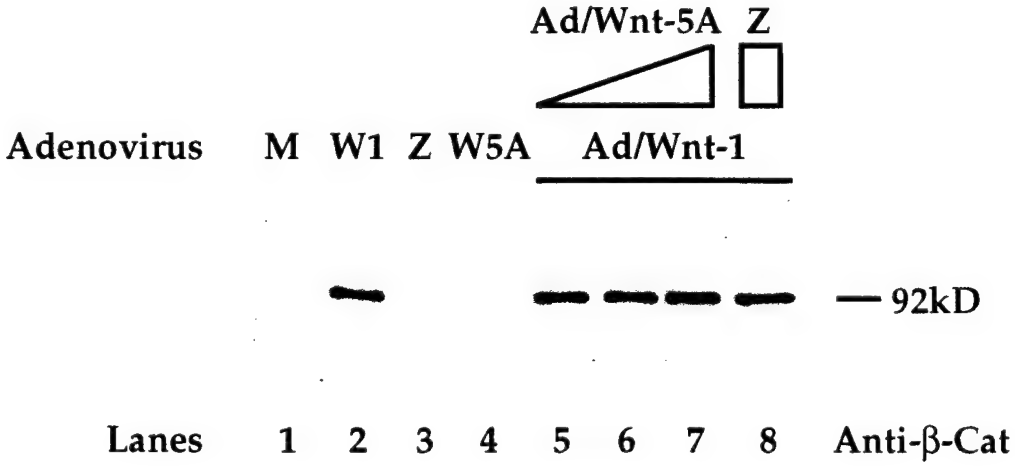


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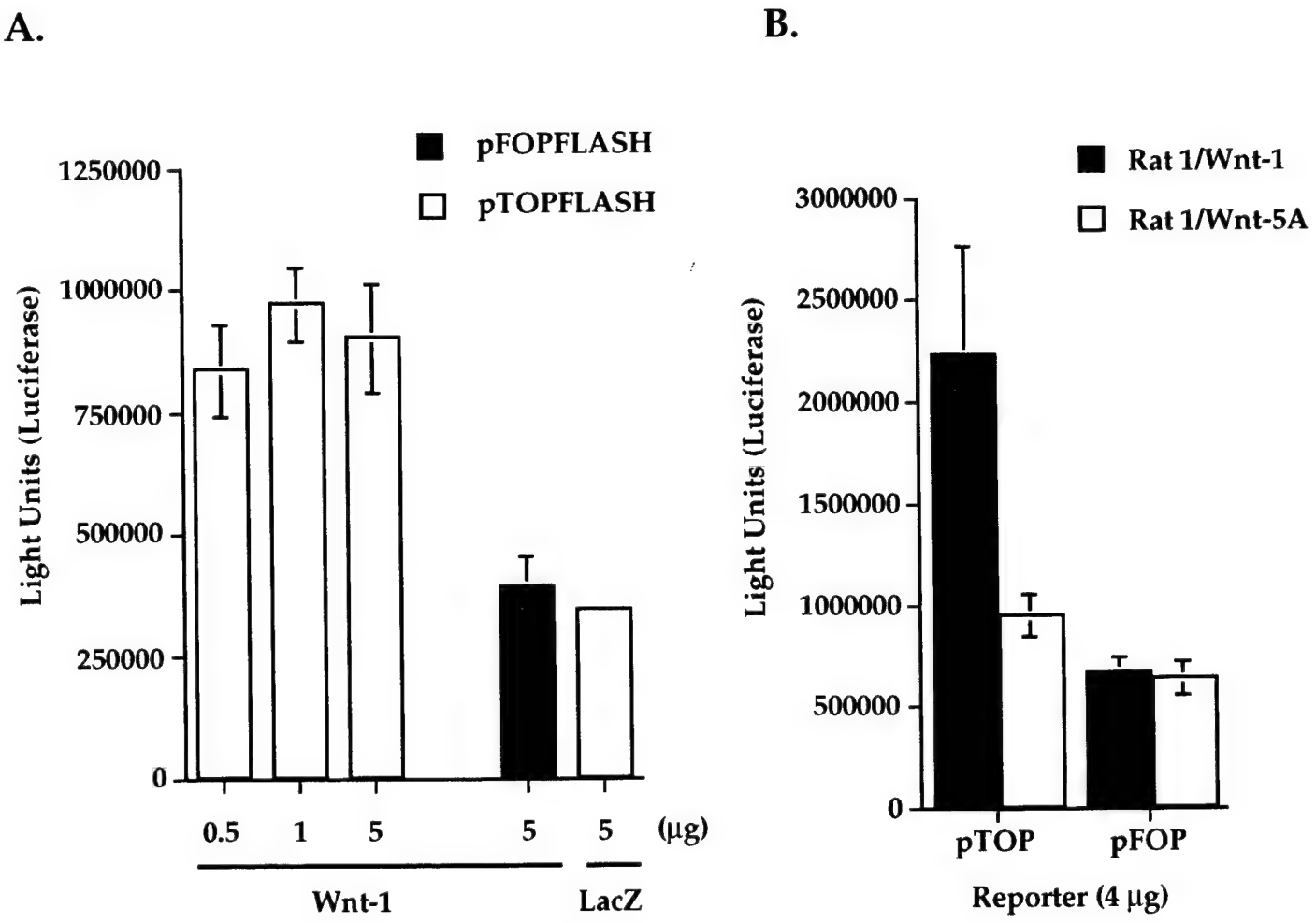
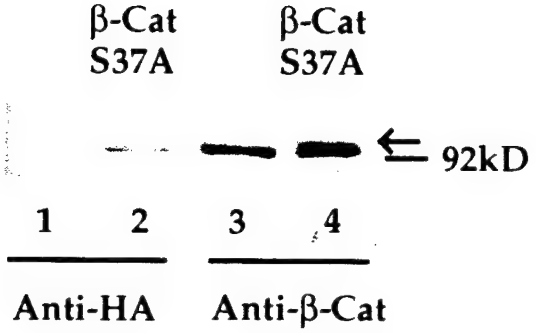
Figure 6



Chapter V

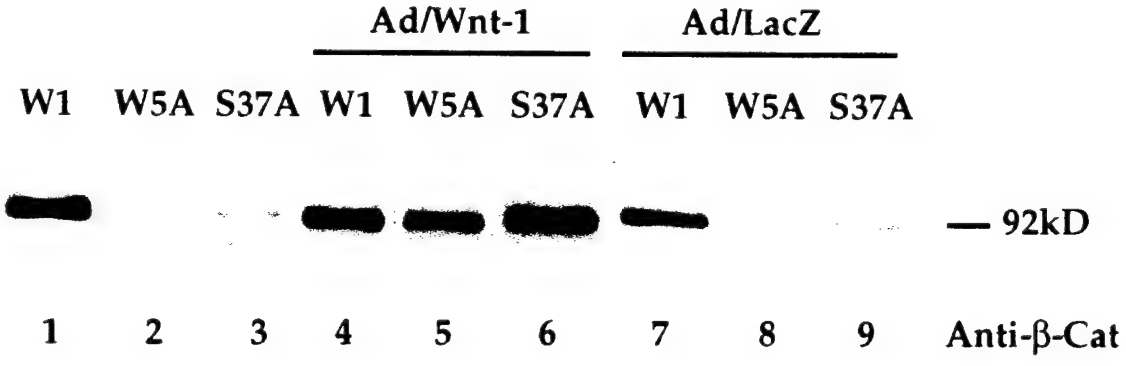
Figure 7

A.



B.

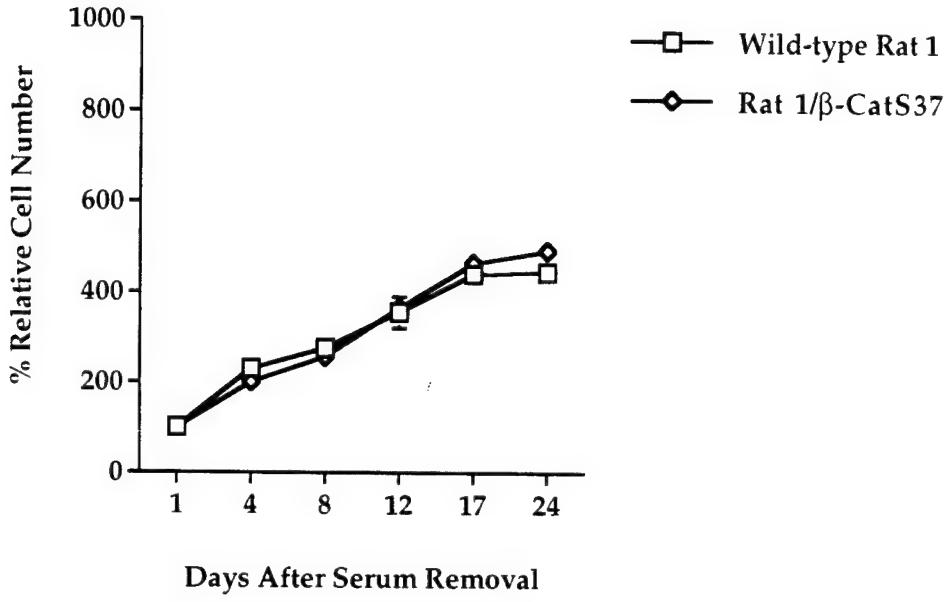
Cytosolic Fractions



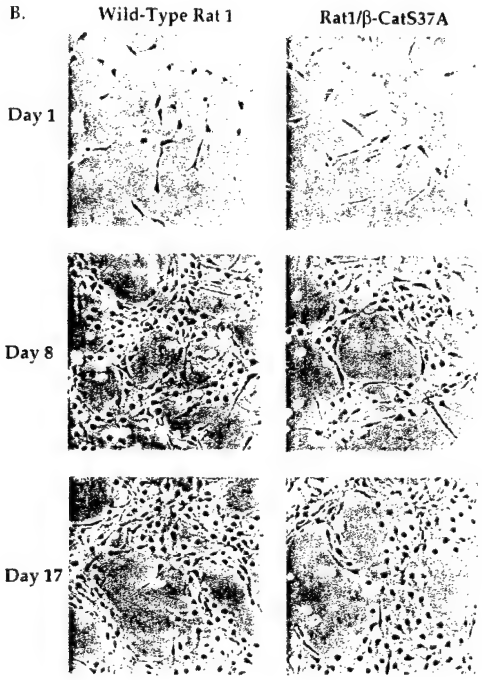
Chapter V

Figure 8

A.

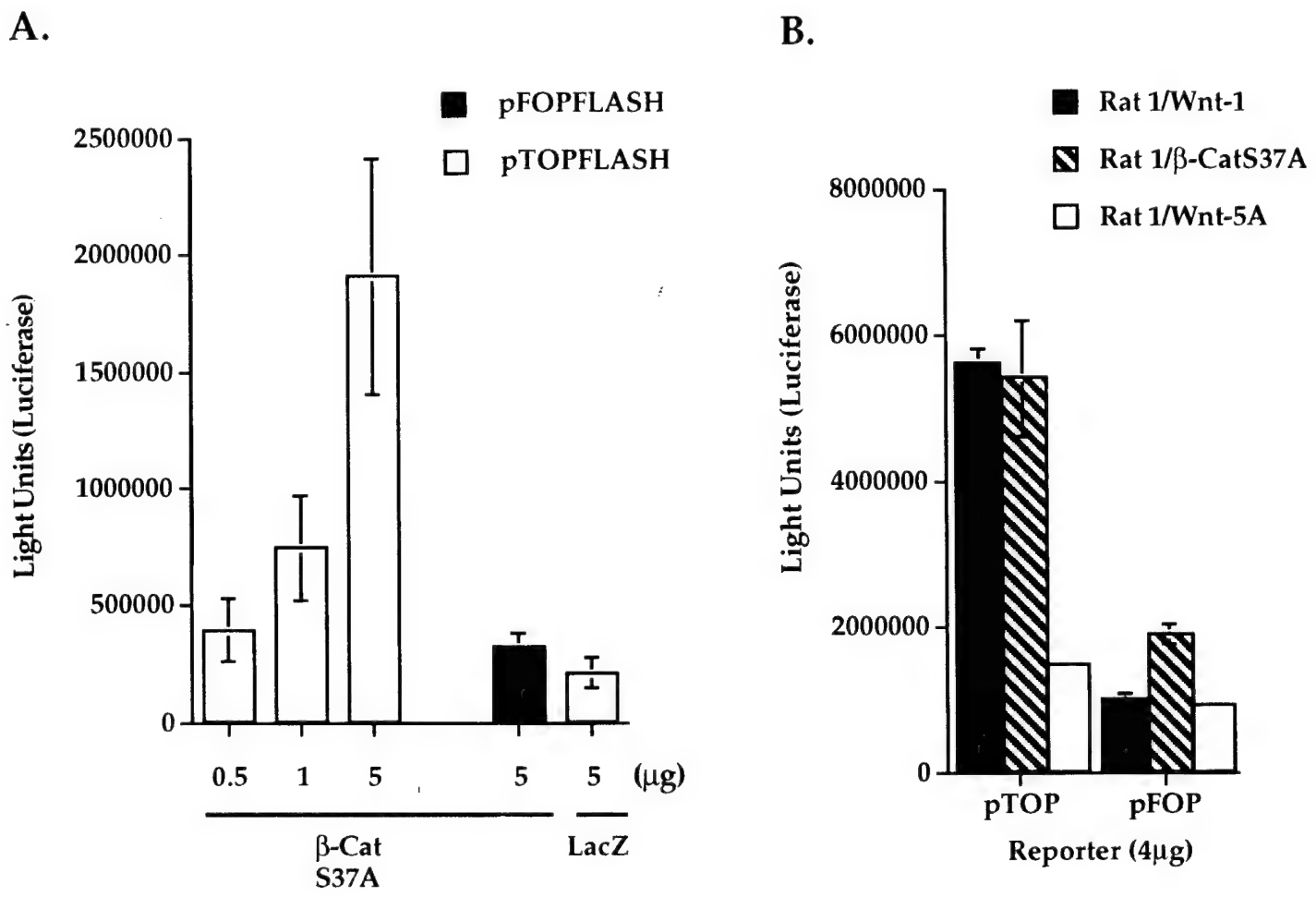


B.



Chapter V

Figure 9



VI. Wnt as a Morphogen

Notch4 and Wnt-1 proteins function to regulate branching morphogenesis of mammary epithelial cells in opposing fashion.

Introduction

The development of the murine mammary gland involves an intricate sequence of proliferative, morphogenetic and differentiative events, which gradually results in the formation of an arborized tree-like structure of epithelial ducts. Postnatal development of the mammary gland is influenced by gonadal hormones, with distinct developmental stages occurring during puberty, estrous, pregnancy and lactation. At birth, the mammary epithelial ducts have few side-branches. During puberty, the epithelial ducts rapidly elongate and branch, and give rise to a highly organized epithelial structure with terminal end buds and lateral buds. The terminal end buds are the major sites of proliferation, whereas the lateral buds differentiate into alveoli during each estrous cycle. During pregnancy, the alveoli rapidly increase in size and number resulting in the development of fully differentiated lobules, which will produce milk at lactation. The mammary gland remodels after lactation ceases, and this process is characterized by the involution of the secretory lobules and regression to the ductal tree observed at puberty (review in Daniel and Silberstein, 1987; Pitelka *et al.*, 1973; Russo *et al.*, 1989).

Mesenchymal-epithelial and epithelial-epithelial interactions are essential in the regulation of growth and development of the murine mammary gland. Peptide growth factors, such as Epidermal growth factor (EGF) [159], Fibroblast growth factors (FGF) [160], Hepatocyte growth factor (HGF), Insulin-like growth factor II (IGF-II) [161], Neuregulin (NRG) [162], and Transforming growth factor–β (TGF–β) [163,164], have been implicated as regulators of mammary gland development based on their expression patterns and, in some cases, on their abilities to affect the development of the mammary gland. HGF (or scatter factor) is expressed in the mammary mesenchyme during ductal branching, whereas its tyrosine kinase receptor c-met is expressed in the mammary epithelial ducts at all stages [165]. HGF can promote branching morphogenesis of the mammary ductal tree [162,165-167] in several experimental settings. TGF–β1 is expressed in the epithelial compartment of the mammary gland at all stages, except during lactation [163,168]. In vivo, TGF–β1 has been shown to inhibit ductal out-growth from the mammary end buds [164,169]. In vitro however, TGF–β1 has been shown to induce opposite effects depending on its concentration. TGF–β1 at high concentrations (0.5-5 ng/ml) inhibit ductal elongation and branching of TAC-2 mammary epithelial cells, whereas at low concentrations (5-100 pg/ml) it is able to stimulate these biological processes [170].

The Wnt family of secreted growth factors are also implicated as regulators of the developing mouse mammary gland [66]. *Wnt* genes are expressed during ductal development of the gland (*Wnt*-2, *Wnt*-5a, *Wnt*-7 and *Wnt*-10b) and during lobular development at pregnancy (*Wnt*-4, *Wnt*-5b and *Wnt*-6), and the expression of most *Wnt* transcripts is down regulated during lactation [72,74]. This pattern of expression during periods of morphogenesis has led to a proposed role for *Wnt* genes in morphogenetic events during mammary gland development. *Wnt* gene expression has been documented in both the stromal and epithelial compartments of the mammary gland, raising the possibility of involvement in both stromal-epithelial and epithelial-epithelial interactions [73,74]. The *Wnt*-1 gene is not normally expressed within the mouse mammary gland, however its expression can contribute to tumorigenesis when activated by insertion of mouse mammary tumor virus (MMTV) proviral DNA in MMTV-induced mammary tumors [7]. Mammary gland tumors develop in transgenic mice where ectopic *Wnt*-1 gene expression is controlled by the MMTV promoter; these mice display hyperplasia of the mammary epithelium and an increased incidence of tumors [8].

The *Notch4* gene was also identified as a frequent target for insertional activation by MMTV proviral DNA in MMTV-induced mammary gland tumors [171,172]. The *Notch4* gene encodes for a large transmembrane receptor protein [173,174]. The int-3 oncoprotein is activated by MMTV insertion and corresponds to a truncated form of Notch4 which has most of its extracellular domain deleted [173]; this mutated version of Notch4 will be referred to as Notch4(int-3). In contrast to *Wnt*-1, expression of the *Notch4(int-3)* oncogene as a transgene in the mouse mammary gland results in impaired development of the mammary gland which no longer generates a tree-like structure of epithelial ducts. Instead, a hyperproliferative mass of undifferentiated epithelial cells is observed near the nipple, from which undifferentiated mammary carcinomas rapidly develop [175].

The aim of this study was to define the roles of both Wnt and Notch signaling in mammary gland ductal morphogenesis. Using a previously described model in which TAC-2 mammary epithelial cells grown in collagen gels form branching cords or tubules in response to HGF or TGF–β1 [166,170], we demonstrate that activation of the Wnt and Notch signaling pathways has opposite effects on branching morphogenesis. Wnt-1 acts to induce branching morphogenesis whereas Notch4(int-3) inhibits branching morphogenesis by either HGF or TGF–β. Wnt-1 has the capacity to overcome the Notch4(int-3) mediated inhibition of branching morphogenesis.

Results

When suspended in collagen gels, TAC-2 mammary epithelial cells form small slowly growing colonies with a morphology ranging from irregular shaped cell aggregates to poorly branched structures. Under these same conditions, TAC-2 cells grown in the presence of either HGF or TGF–β1 develop an extensive network of branching cords that consist of elongated epithelial cords or tubules with multiple branch points [166,170]. This TAC-2 cell phenotype is thus reminiscent of the branching morphogenesis of epithelial ducts in the mammary gland and provides an experimental model to study the roles of growth factors and receptors in the development of the mammary gland. To investigate the role of Wnt and Notch signaling in mammary epithelial cell growth and morphogenesis we ectopically expressed either the Wnt-1 or activated Notch4(int-3) oncoproteins and analyzed their effects on branching morphogenesis of TAC-2 cells. The results described below represent those found with several independently derived cell lines, including independent lines that were programmed to express proteins using a different promoter, as noted in the text.

Wnt-1 stimulates TAC-2 cell branching morphogenesis.

The biological activity of Wnt proteins was evaluated by generating TAC-2 cells ectopically expressing a *Wnt*-1 cDNA. TAC-2 cell lines programmed to express *Wnt*-1 (TAC-2 Wnt-1) were generated using the retroviral vector pLNCX to drive *Wnt*-1 expression from the CMV promoter . As a control, TAC-2 cells were generated that were programmed to express LacZ (TAC-2 LacZ). To evaluate the expression levels of Wnt-1 proteins in the cell lines generated, the *Wnt*-1 cDNA was fused at the carboxy terminus to the haemagglutinin-epitope (HA) tag, allowing us to detect Wnt-1 proteins in immunoblot analysis using the anti-HA monoclonal antibody (Fig. 1). Cell extracts from TAC-2 cell lines contained Wnt-1 proteins (Fig. 1) that migrated as a series of proteins with molecular weights between 41 and 45 kD, due to differential glycosylation. The ectopic expression of Wnt-1 proteins in TAC-2 Wnt-1 cells can be significantly increased by treating cells with sodium butyrate (2mM), which enhances transcription of the CMV promoter (Fig. 1). In order to evaluate the effects of different protein levels on branching morphogenesis of TAC-2 cells, experiments were done either in the presence or absence of sodium butyrate. We found that addition of sodium butyrate to the TAC-2 branching morphogenesis assay did not alter or enhance the TAC-2 cell phenotypes described below.

TAC-2 cells programmed to express LacZ give rise to small colonies with poorly branched cords when grown in collagen gels for four days (Fig. 2A). Addition of either HGF (20 ng/ml) or TGF–β2 (50 pg/ml) to the culture induces pronounced changes in colony morphology, resulting in the formation of long branching cords or tubules (Fig. 2B and 2C). We utilized TGF–β2 in our assays, which we found has an identical activity as TGF–β1 in the induction of branching morphogenesis of TAC-2 cells [170]. When TAC-2 cells are programmed to express Wnt-1 proteins, cell colonies form cords with moderate branching even in the absence of exogenous growth factors (compare Fig. 2A and 2D). When TAC-2 Wnt-1 cells are grown in the presence of either HGF or TGF–β2 (Fig. 2E and 2F), a highly extensive branching network is observed. This network of epithelial tubules is significantly more extensive when compared to control TAC-2 LacZ cells grown under identical conditions. An identical phenotype was observed in TAC-2 cell lines programmed to express either a non-epitope tagged *Wnt*-1 cDNA or a HA epitope tagged *Wnt*-1 cDNA transcribed from a SV40 based retroviral vector (data not shown). Thus, Wnt-1 activity was confirmed in at least three independently produced TAC-2 cell lines.

The morphological analysis of TAC-2 cell cultures suggested that Wnt-1 cooperates with either HGF or TGF–β2 in the induction of branching morphogenesis. To characterize combined effects of Wnt-1 and HGF, quantitative evaluation of cord length and the number of branch points was conducted (Fig. 3A and 3B). Both analyses showed that the branched network formed by TAC-2 Wnt-1 cells grown under control conditions was comparable to that found for HGF-treated TAC-2 LacZ cultures. When

TAC-2 Wnt-1 cells are grown in the presence of HGF, both cord length and number of branch points is significantly greater than the combined values for TAC-2 Wnt-1 cells without HGF and TAC-2 LacZ cells grown with HGF (Fig. 3A and 3B). Thus, Wnt-1 and HGF act in a cooperative fashion to induce branching morphogenesis of TAC-2 cells.

To determine whether the effects of Wnt-1 on TAC-2 cell branching morphogenesis are due to effects on the growth characteristics of TAC-2 cells we compared the growth of the TAC-2 cell lines generated. TAC-2 cells were plated at different densities on collagen coated dishes, either in the presence or absence of HGF, and viable cell numbers were determined either two or six days after plating. No significant differences in cell number were found between control TAC-2, TAC-2 LacZ, or TAC-2 Wnt-1 cell lines grown in either the presence or absence of HGF (data not shown). When grown under these conditions, TAC-2 Wnt-1 cells and control TAC-2 cells both displayed contact inhibition at confluence and had similar morphological characteristics. Hence, the effects of Wnt-1 on TAC-2 branching morphogenesis are not correlated with mitogenic activity of Wnt-1 and are dependent on growth in three dimensional collagen gels. Identical results were obtained with TAC-2 cell lines that were programmed to express Wnt-1 using a SV40 based retroviral vector.

To further characterize morphogenetic activities of Wnt-1 proteins, we analyzed TAC-2 cells induced to form cyst structures in collagen gel cultures. When TAC-2 LacZ cells are grown in collagen gels in the presence of hydrocortisone and cholera toxin, they form spheroidal cysts enclosing a widely patent lumen, as previously observed with non-transfected TAC-2 cells [166](Fig. 4A, C, E). In contrast, under the same experimental conditions, TAC-2 Wnt-1 cells form branching structures (Fig. 4B, D, F). This clearly indicates that Wnt-1 expression modifies the spatial arrangement of TAC-2 cells and therefore has a morphogenetic effect.

Notch4(int-3) inhibits TAC-2 cell branching morphogenesis.

Notch4 activity in branching morphogenesis was evaluated by expressing an activated form of the Notch4 receptor, Notch4(int-3), in TAC-2 cells. TAC-2 cell lines programmed to express the Notch4(int-3) proteins were generated using the retroviral vector pLNCX and will be referred to as TAC-2 int-3. Notch4(int-3) was HA-epitope tagged at the carboxy terminus to allow detection of ectopically expressed proteins. Immunoblot analysis using anti-HA antibodies detected Notch4(int-3) proteins that migrate with an approximately molecular weight of 60 kD, corresponding well to their predicted molecular weight (Fig. 1).

When TAC-2 int-3 cells are grown in collagen gels and incubated in the presence of either HGF or TGF–β2 (Fig 2H and 2I), cell colonies no longer form elongated cords like control cultures (Fig 2B and 2C). Instead, HGF- or TGF–β2-treated TAC-2 int-3 cell colonies form small aggregates or structures with rudimentary branches which are similar in appearance to those formed by either TAC-2 LacZ or TAC-2 int-3 colonies grown in the absence of HGF or TGF–β2 (Fig 2A and 2G). An identical phenotype was observed in at least three independently produced cell lines, including TAC-2 cells programmed to express a non-epitope tagged *Notch4(int-3)* cDNA or a HA epitope tagged *Notch4(int-3)* cDNA transcribed from a SV40 based retroviral vector (data not shown). Interestingly, we found that a smaller percentage of TAC-2 int-3 cells give rise to colonies in collagen gels with respect to TAC-2 LacZ cells (230±32 colonies/cm2 in TAC-2 int-3 cells versus 795±114 colonies/cm2 in TAC-2 LacZ cells), which suggests that Notch4(int-3) expression reduces plating (colony formation) efficiency in collagen gels. Accordingly, to avoid overestimating the inhibition of HGF-induced cord elongation in TAC-2 int-3 cells, the quantitative analysis of cord length and branching was carried out on a per colony basis, rather than on a per field basis (see Materials and Methods). This analysis demonstrated that, despite the fact that colonies formed by TAC-2 int-3 cells are slightly more elongated and branched than those formed by TAC-2 LacZ cells, their morphogenetic response to HGF is markedly decreased (Fig. 3C and 3D).

We analyzed the growth characteristics of the TAC-2 int-3 cell line, as described for TAC-2 Wnt-1 cells. TAC-2 int-3 cells plated on collagen coated dishes, either in the presence or absence of HGF, displayed no significant differences in cell number, morphology, or growth post-confluence when compared with TAC-2 controls (data not shown). Identical results were obtained with TAC-2 cell lines programmed to express Notch4(int-3) using a SV40 based retroviral vector. Hence, the effects of Notch4(int-3) on TAC-2 branching morphogenesis are not correlated to changes in the growth properties in the cells.

The carboxy terminus of the Notch4(int-3) is not required for activity.

The Notch4(int-3) oncoprotein, has most of the extracellular domain of Notch4 deleted and consists of the transmembrane and intracellular domains. To investigate which region(s) of Notch4(int-3) proteins are required and sufficient for activity, a series of Notch4(int-3) deletion mutants were generated (schematized in Fig. 5A). Four Notch4(int-3) deletion mutants were made and designated ΔNT (deletion of the amino terminal domain), ΔCDC (deletion of cdc10 repeat domain), ΔCT (deletion of the carboxy terminal domain) and ΔNTΔCT (N-terminal and C-terminal deletion) (Fig. 5A). All four mutant int-3 cDNAs were HA-epitope tagged at their carboxy termini and TAC-2 cell lines programmed to express each deletion mutant were generated using the retroviral vector pLNCX. Immunoblot analysis using anti-HA monoclonal antibodies demonstrated expression of Notch4(int-3) deletion proteins of appropriate molecular weight in each respective cell line (Fig. 5B). The ΔNTΔCT Notch4(int-3) deletion protein with predicted molecular weight of 25 kD co-migrates with a non specific anti-HA antibody background band (Fig. 5B), however, was detected in a separate analysis of both untreated TAC-2 ΔNTΔCT cells or cells treated with sodium butyrate (Fig. 5C). TAC-2 cell lines expressing the four different Notch4(int-3) deletion mutants were grown in collagen gels as described above, and the ability of each Notch4(int-3) deletion mutant to inhibit HGF induced branching morphogenesis of TAC-2 cells was analyzed. As shown in Fig. 6, TAC-2 cells expressing either ΔNT (Fig. 6A and 6B), ΔCDC (Fig. 6C and 6D) or ΔNTΔCT (Fig. 6G and 6H) are responsive to HGF induced branching morphogenesis. In contrast, when grown in the presence of HGF, ΔCT expressing TAC-2 cells (Fig. 6E and 6F) display an identical phenotype as the TAC-2 int-3 cells. Hence, the carboxy terminus of the Notch4(int-3) is not required for Notch-mediated inhibition of TAC-2 branching morphogenesis. Thus, in this assay the activity of the Notch4(int-3) oncoprotein can be conferred by the amino terminus and cdc10 repeats.

Branching morphogenesis in cells co-expressing Wnt-1 and Notch4(int-3) oncoproteins.

The activation of the Wnt-1 and Notch signaling pathways resulted in opposite effects on HGF- or TGF–β2-induced branching morphogenesis of TAC-2 cells. To explore the interactions between these two signaling pathways, we investigated the effect of simultaneous expression of both Wnt-1 and Notch4(int-3) proteins on TAC-2 branching morphogenesis. The above described TAC-2 LacZ and TAC-2 int-3 cell lines, which were generated with the pLNCX expression vector, were now also programmed to express Wnt-1 using the retroviral vector pLHTCX. This vector drives gene expression from the CMV promoter and contains the hygromycin resistance gene. In this fashion, four additional TAC-2 cell lines were generated that were named TAC-2 LacZ/ctr, TAC-2 LacZ/Wnt-1, TAC-2 int-3/ctr and TAC-2 int-3/Wnt-1 (where ctr denotes control empty pLHTCX vector). To determine appropriate protein expression in each of these four cell lines, immunoblot analysis showed Notch4(int-3) and Wnt-1 proteins were expressed as expected and at levels similar to those previously found to confer activity (data not shown). Each of the four cell lines were grown in collagen gels to determine their ability to undergo HGF- or TGF–β2-induced branching morphogenesis (Fig. 7). This assay was repeated three times with similar results. Doubly infected control cells TAC-2 LacZ/ctr (Fig. 7A, B, C) remained responsive to both HGF and TGF–β demonstrating that two rounds of drug selection did not affect the phenotype of the TAC-2 cell lines. As observed previously for TAC-2 Wnt-1 cells, TAC-2 LacZ cells that are now programmed to express Wnt-1 (Fig. 7D, E, F) form small colonies that undergo modest branching even in the absence of HGF or TGF–β2; these cells form extensive elongated branches when grown in the presence of HGF or TGF–β2. The activity found for Notch4(int-3), that is the inhibition of HGF- and TGF–β-induced branching morphogenesis, was also found in the TAC-2 int-3/ctr cell line (Fig. 7G, H, I). Wnt-1 and Notch4(int-3) co-expressing cells, TAC-2 int-3/Wnt-1, are able to form colonies displaying branching and elongation and have an appearance similar to that of TAC-2/LacZ/Wnt-1 cells (Fig. 7J). An examination of several fields reveal that TAC-2 int-3/Wnt-1 cells displayed increased responses when treated with either HGF or TGF–β, thus these cells now regain responsiveness to these factors (Fig. 7K, L). Our results indicate that Notch activation attenuates responsiveness of TAC-2 cells to both HGF and TGF–β and that Wnt-1 can override the Notch activity in TAC-2 cells.

Discussion

In this study, we have detailed a regulatory hierarchy involved in the branching morphogenesis of TAC-2 mammary epithelial cells. This regulation includes four distinct signaling pathways; the Wnt, Notch, HGF, and TGF–β signaling cascades. Using an <u>in vitro</u> model that reflects the branching morphogenesis exhibited during mammary gland development we have assessed the potential interactions between several different signaling pathways. This approach has allowed us to establish the relationships

between these pathways. One remarkable feature of the regulation of branching morphogenesis we describe is its similarity to the regulatory pathways leading to morphogenetic events during Drosophila development.

Wnt proteins as branching morphogens in the mammary gland.

Formation of branching cords is induced in collagen gel cultures of TAC-2 cells by the addition of either HGF or low concentrations of TGF–β. Wnt-1 proteins induce moderate branching and elongation of TAC-2 cell tubules in the absence of added HGF or TGF–β2. The extent of Wnt-1 induced branching morphogenesis of TAC-2 cells is comparable to the induction by either HGF or TGF–β2. Our evidence suggests that Wnt-1 acts as a morphogen in this capacity. First, Wnt-1 induces a change in a morphogenetic event, the formation of branched epithelial tubules or cords. Second, Wnt-1 does not appear on its own to alter the growth properties of the TAC-2 cells. Finally, Wnt-1 can induce branching in an environment where cysts typically form; that is, in hydrocortisone and cholera toxin treated cultures. Such cultures form spheroidal cysts enclosing a widely patent lumen. In this environment, peptide growth factors that display mitogenic and not morphogenetic properties would increase the size of the cyst but the spheroidal structure would be maintained. In contrast, Wnt-1 alters the morphogenetic behavior in such a way that new branch points are formed and the structures take on a tubular morphology.

Several Wnt proteins are expressed in the mammary gland during periods of morphological changes of the ductal epithelium (*Wnt*-2, *Wnt*-5a, *Wnt*-7 and *Wnt*-10b) (Gavin and McMahon, 1992; Weber-Hall *et al.*, 1994). Ectopic expression of Wnt-1 *in vivo* suggests a role for Wnt proteins in cell proliferation during mammary gland development; however, morphogenetic changes also occur in response to Wnt-1. It is has been proposed that the Wnt-1 expression mimics the activity of endogenous mammary gland Wnt proteins. A transgenic line driving expression of *Wnt*-1 to the mammary gland displays a hyperplastic phenotype, indicative of increased proliferation [8]. In addition, both virgin females and males display a marked increase in the number of terminal branches, and in fact resemble the hormonally stimulated glands normally observed in pregnant animals. Tissue reconstitution experiments in which *Wnt*-1 is ectopically expressed in mammary epithelium also result in a hyperplastic gland where duct epithelium show abundant fine side-branches, suggesting that Wnt-1 may instruct the epithelium to form branches [176]. This phenotype is most likely not simply a consequence of proliferation as it is not seen with a variety of other oncogenic proteins which when ectopically expressed in the mammary gland induce hyperplasia without increasing branching [177].

The Wnt signal transduction pathway is mediated in part through β–catenin, a protein associated with cadherins, and which is necessary for the adhesive functions of adherens junctions [93]. Wnt-1 signaling results in stabilization of the cytoplasmic pool of β–catenin [124,127], which then can associate with downstream targets in the cytoplasm to transduce signals that lead to regulation of target gene expression [79,147]. TAC-2 cells programmed to express Wnt-1 in fact display increased levels of cytosolic β–catenin, when compared to TAC-2 LacZ cells (our unpublished data). This stabilization may be regulated by the phosphorylation of β–catenin on serine/threonine residues, possibly by glycogen synthase kinase 3 (GSK-3) [132]. Recent evidence has demonstrated the importance of β–catenin/cadherin interactions in regulating cell adhesion, cell migration and epithelial phenotype in embryonic development [55]. The activation of β–catenin by Wnt-1 induced signaling may result in changes of the adhesive and migratory characteristics of mammary epithelial cells and consequently affect ductal morphogenesis of TAC-2 cells.

Cooperative interactions between Wnt-1 and HGF or TGF–β.

In response to the combined effects of Wnt-1 and HGF, TAC-2 cells form a network of elongated and branching tubules that is far more extensive than the branching cords observed when TAC-2 cells are grown in the presence of Wnt-1 or HGF singularly. We propose that the combined effect of HGF addition and Wnt-1 expression is not the result of the sum of their independent activities on branching morphogenesis, but that Wnt signaling synergizes with the HGF/c-met tyrosine kinase pathway. The possibility that Wnt proteins cooperate in vivo with the HGF/c-met pathway in the regulation of mammary morphogenesis is supported by the overlapping temporal patterns of *Wnt* genes and HGF/c-met expression [72,74,167].

One potential area where these two signaling pathways could converge might be through their effects on the catenin and cadherin proteins. The cooperation between Wnt-1 and HGF may be explained

by their combined activation of β–catenin. β–catenin has been detected in a complex with the EGF receptor and can be phosphorylated in response to EGF and HGF [178,179]. In addition, the Ras pathway is essential for the biological activity induced by HGF/c-met [180] and β–catenin has been demonstrated to be a substrate for tyrosine kinases and to become tyrosine phosphorylated in cells expressing activated Src and Ras [181]. Another catenin-like protein, p120, which was identified as a substrate of Src and several receptor tyrosine kinases, interacts with the cadherin-β–catenin complex and may participate in regulating the adhesive function of cadherins [182]. EGF is also able to stimulate branching morphogenesis of TAC-2 cells, although not to the same extent as HGF, whereas NGF, bFGF, IGF-II and KGF can not [166]. These activities correlate with the reported phosphorylation of β–catenin by the EGF and HGF signal transduction pathways [179]. It is yet unclear how tyrosine phosphorylation of β–catenin might regulate its activity. Tyrosine phosphorylated β–catenin is found in a detergent soluble pool [178,183], which may reflect specific phosphorylation of a free pool of β–catenin. Since both Wnt-1 and HGF signaling can converge on β–catenin, it is therefore possible that the observed cooperation between HGF and Wnt-1 is due to their combined action on β–catenin activity.

Wnt-1, HGF, and TGF–β could induce branching morphogenesis by regulating the adhesive and migratory properties of TAC-2 cells through modulation of extracellular matrix components and their interaction with their receptors. Since HGF has been demonstrated to decrease adhesion of TAC-2 cells to collagen and to enhance the deposition of type IV collagen it is also possible that the observed cooperation between HGF and Wnt-1 is due to their combined effect on cell-substrate adhesion. TGF–β signaling involves receptors with serine/threonine kinase activity which are known to regulate the synthesis and degradation of extracellular matrix molecules and to induce matrix organization. Induction of branching morphogenesis by TGF–β could be mediated by a remodeling of extracellular matrix components and cell-substrate interactions. The Wnt signal transduction pathway may also regulate cell-substrate interactions, and the combined activity of both Wnt-1 and TGF–β may explain their cooperative activities on the branching morphogenesis of TAC-2 cells.

In *Drosophila*, wingless (*wg*) and the TGF–β homologue *Decapentaplegic* (*Dpp*) have been shown in some cases to act in combination to regulate gene transcription during inductive events. In particular, Wg and Dpp have been shown to act in combination during limb development [184,185] and to induce *Ultrabithorax* expression during endoderm induction [44]. Wnt-1 and TGF–β signaling may similarly converge to affect gene transcription during branching morphogenesis in the mouse mammary gland.

Notch inhibits branching morphogenesis of mammary epithelial cells.

We demonstrate that Notch activation inhibits both the HGF and TGF–β induced branching morphogenesis of TAC-2 mammary epithelial cells. The precise mechanism of this inhibition is unclear. Activation of Notch signaling has been demonstrated to inhibit or alter the cell fate commitment or differentiation of a variety of different cell types [186,187]. For instance, *C. elegans* Lin-12 controls cell fate decisions during gonadogenesis, *Drosophila Notch* acts to control cell fate during neuroblast and photoreceptor cell differentiation, an activated *Xenopus Notch* can affect epidermal and neural crest cell development, and an activated mouse *Notch*1 can control cell fate during myogenesis and neurogenesis of cultured mouse cells [186,187]. Transgenic mice that use the MMTV viral promoter to express the Notch4(int-3) oncoprotein, the activated form of Notch4, display severely impaired mammary ductal growth [175]. When *Notch4(int-3)* is expressed from the whey acidic protein promoter, whose expression is restricted to the secretory mammary epithelial cells, the differentiation of the secretory lobules of the transgenic animals is profoundly inhibited [188]. These experiments demonstrate that *Notch4(int-3)*, like many other activated *Notch* genes, can act as a regulator of cell fate decisions in the mammary gland of mice. Little is known about the spatial and temporal pattern of *Notch* gene expression in the mammary gland, however, *Notch4* is expressed *in vivo* in the murine mammary gland [172,189]. *Notch* genes may thus regulate the cell fate decisions occurring during mammary gland development that lead to the branched epithelial strutcture of the gland.

We have found that Notch activation can affect the response of TAC-2 mammary epithelial cells to either HGF or TGF–β. Since HGF acts through a tyrosine kinase receptor and TGF–β acts through serine/threonine kinase receptors, the affects of Notch activation may involve more than specific inhibition of a particular signaling cascade. Notch may regulate the competency of TAC-2 cells to respond to several different factors, possibly by shifting TAC-2 cells to a fate that is not predisposed to undergoe branching morphogenesis. This model would be consistent with the proposed activites of Notch proteins in several different organisms. Alternately, these signaling pathways may be controlled by Notch

at a point at which they may converge to induce expression of genes important for branching morphogensis. Recently, the intracellular domain of LIN-12, a *C. elegans* Notch, has been demonstrated to associate with EMB-5, which encodes for a cytoplasmic protein containing a SH2 domain [190]. This finding raises the possibility that the Notch signaling proteins may interact directly with those elicited by tyrosine kinase receptors, such as the HGF receptor (c-met).

We have demonstrated that the domain, carboxy terminal to the cdc10 repeats, of the Notch4(int-3) oncoprotein is not required for biological activity. However, the amino terminal domain and the cdc10 repeats are required for Notch4(int-3) activity. These findings are consistent with previous observed data for other *Notch* genes. The RAM23 domain which is localized between the transmembrane and cdc10 repeats has been demonstrated to be the binding site of CBF-1, a downstream and essential element in Notch signaling [191]. Deletion of the amino terminal domain of Notch4(int-3), which contains the RAM23 domain, may eliminate binding to CBF-1, and hence destroy Notch4(int-3) activity. The region of the LIN-12 protein that includes the RAM-23 domain and cdc10 repeats appears to interact with another downstream and positive regulator, EMB5 [190]. Point mutations and deletions within the cdc10 repeats result in loss of function of Notch proteins [192]. Our data thus indicates that Notch4 may interact and be regulated through similar mechanisms.

Competing influences of Wnt and Notch signaling in branching morphogenesis.

When TAC-2 cells are programmed to express both Wnt-1 and Notch4(int-3), the cells are able to undergo branching morphogenesis. In Wnt-1 and Notch4(int-3) coexpressing TAC-2 cells, branching morphogenesis can be increased by either HGF or TGF–β; that is, the cells regain responsiveness to these factors. The phenotype observed in Wnt-1 and Notch4(int-3) coexpressing cells was similar to that of TAC-2 cells expressing only Wnt-1. The opposite biological activities of Wnt-1 and Notch4(int-3) observed in the TAC-2 cell assay correlate well with the mammary gland phenotype observed in Wnt-1 and Notch4(int-3) transgenic mice that ectopically express these proteins in the mammary gland [175]. Although both oncogenes increase mammary tumor development, Wnt-1 stimulates a hyperplastic phenotype with increased ductal development whereas Notch4(int-3) inhibits ductal development.

Wnt-1 can override the Notch4(int-3)-mediated inhibition of branching morphogenesis providing the first evidence of interaction between these two signaling pathways in vertebrates. The dominance of Wnt-1 over activated Notch we have observed in murine cells parallels the functional relationship proposed for *Drosophila Wnt* (*wingless*) and *Notch* during *Drosophila* development [193]. In this study, genetic analysis suggests a pathway convergence between wingless and Notch signaling resulting in opposing effects during patterning of the developing *Drosophila* wing. Activation of the wingless signal leads to regulation of Notch activity, possibly by *Drosophila* dishevelled, a cytoplasmic protein that is also a positive mediator in the Wnt-1 signal transduction pathway. Analysis using a yeast interaction trap system demonstrated that Dishevelled physically associates with the intracellular domain of Notch. The antagonism between Wnt-1 and Notch4(int-3) seen in branching morphogenesis may also be mediated by common regulators of the two signaling pathways such as Dishevelled.

During mammary gland development, the growth and differentiation of the gland is regulated by mesenchymal-epithelial and epithelial-epithelial interactions. Cells often receive different signals simultaneously and must integrate them in order to take on the correct proliferative, morphogenetic or differentiative response. Notch inhibition of ductal morphogenesis may be an early event in ductal morphogenesis. An attractive mechanism for overcoming Notch and allowing ductal morphogenesis to initiate or progress would be to activate the expression of a *Wnt* gene(s). Wnt could then serve the dual function of suppressing Notch activity and initiating branching morphogenesis. Wnt signaling may then cooperate with other signaling pathways, such as those mediated by HGF and TGF–β, in order to complete branching morphogeneis. Our study thus has revealed complex interactions between the signal transduction pathways of Wnt, Notch, HGF and TGF–β, in regulating the branching morphogenesis of mammary epithelial cells.

Materials and methods

Reagents.

Recombinant human HGF (rhHGF) was provided by Genentech, Inc. (San Francisco, CA). Recombinant TGF–β2 was provided by Dr. G. Gunderson (Columbia University, New York, NY). Rat tail collagen solution was obtained from Upstate Biotechnology Inc. (Lake Placid, NY). Anti-HA monoclonal

antibody (12CA5) was from Berkeley Antibody Co. (Richmond, CA) and HRP-conjugated sheep anti-mouse immunoglobulin G from Amersham (Arlington Heights, IL).

cDNA clones.

The murine *Notch4(int-3)* cDNA corresponds to a truncated *Notch4* cDNA; residues 4551 to 6244 of *Notch4* (Uyttendaele et al. 1996). An oligonucleotide encoding the haemagglutinin (HA) antigenic determinant was appended to the 3' end of the *Notch4(int-3)* and *Wnt*1 cDNA's. Eighteen codons were added that specify the amino acid sequence SMAYPYDVPDYASLGPGP, including the nine residue HA epitope (underlined). HA-tagged *Notch4(int-3)* and *Wnt*1 cDNAs were created by subcloning each cDNA into Bluescript KS (Stratagene) with the coding region of the HA epitope situated downstream of the newly inserted cDNA. These two sequences were made co-linear by "loop-out" mutagenesis using oligonucleotides designed to eliminate the stop codon and non-coding 3' sequence of the *Notch4(int-3)* and *Wnt*1 cDNAs. Oligonucleotides used in this procedure are as follows;

Notch4(int-3): CGG TTG TAA GAA ATC TGA ACT CCA TGG CCT ACC CAT ATG

Wnt-1: CGC GCG TTC TGC ACG AGT GTC TAT CCA TGG CCT ACC C .

The 5' end of each oligo is complementary to the C-terminus of *Notch4(int-3)* or *Wnt*1 cDNA and their 3' ends anneal to HA epitope-encoding sequence (underlined). Mutagenesis was done with the Muta-Gene*in vitro* mutagenesis kit (Bio Rad, Richmond, CA). The presence of each fusion was confirmed by DNA sequencing. *Notch4(int-3)* cDNA deletion mutants were generated from the epitope-tagged *Notch4(int-3)* construct by restriction enzyme cloning, and were named ΔNT, ΔCDC, ΔCT and ΔNTΔCT. The ΔNT deletion mutant corresponds to nucleotides 4921 to 6244 of the *Notch4* sequence. The ΔCDC deletion mutant corresponds to nucleotides 4551 to 4864 and to nucleotides 5706 to 6244 of the *Notch4* sequence. The ΔCT deletion mutant corresponds to nucleotides 4551 to 5718 of the *Notch4* sequence. The ΔNTΔCT deletion mutant corresponds to nucleotides 4921 to 5718 of the *Notch4* sequence.

Cell culture.

The TAC-2 cell line was derived from NMuMG cells as described previously [166]. TAC-2 cell were grown on collagen-coated dishes in Dulbecco's modified Eagle's medium (DMEM, GIBCO) supplemented with 10% fetal calf serum (FCS, GIBCO). The Bosc 23 retrovirus packaging cell line (Pear et al. 1993) was obtained from Dr. W. Pear (MIT, MA) and grown in DMEM containing 10% FCS. Both culture media were supplemented with penicillin (500 i.u./ml) and streptomycin (100ug/ml). Both cell lines were grown at 37°C in 8% CO_2.

Cell line generation.

HA-tagged cDNAs were inserted into the retroviral vector pLNCX [114] wherein neomycin phosphotransferase (neo) expression is controlled by the murine leukemia virus LTR, and cDNA transcription is controlled by the cytomegalovirus (CMV) enhancer/promoter. The retroviral vector pLHTCX was derived from pLNCX, however the neo gene is replaced by a hygromycin-resistence gene. Populations of TAC-2 cells, expressing either HA-tagged *Notch4(int-3)* or *Wnt*1 cDNA, were prepared by retroviral infection. Recombinant retroviruses were generated by transiently transfecting constructs into the BOSC 23 cell line by calcium phosphate co-precipitation, as previously described [111]. Retroviral infection of TAC-2 cells was done by culturing cells with viral supernatants collected from transfected BOSC 23 cells two day post-transfection. Infections were done in the presence of 4 μg/ml polybrene for 12 hours after which medium was replaced to DMEM + 10% FCS. One day post-infection the culture medium was replaced to DMEM + 10% FCS containing 500 μg/ml Geneticin (GIBCO BRL Life Technologies, Grand Island, NY) or 200 μg/ml hygromycin B (Sigma Chemical Co.). Colonies appeared 5 days later and were pooled into medium containing 250 μg/ml Geneticin or 200 μg/ml hygromycin B. These resultant populations, each comprised of at least 50 clones, were used in assays described below.

Collagen cell culture assays.

TAC-2 cell lines were harvested using trypsin-EDTA, centrifuged, and embedded in three-dimensional collagen gels as previously described [166]. Briefly, 8 volumes of rat tail collagen solution (approximately 1.5 mg/ml) were mixed with 1 volume of 10x minimal essential medium (GIBCO) and 1 volume of sodium bicarbonate (11.76 mg/ml) in a sterile tube kept on ice to prevent premature collagen gellation. TAC-2 cells were resuspended in the cold mixture at cell densities of 2 or 4 x 10^4 cells/ml and 0.5 ml aliquots were dispensed into 16-mm wells of 24 multiwell plates (Becton Dickinson Labware). After the collagen mixture had gelled, 1ml of complete medium (DMEM + 10% FCS) with or without HGF or TGF-β2 was added to each well. TAC-2 collagen gel cultures were initially done in the presence and absence of 2mM sodium butyrate, but since no difference in phenotypes was observed, the sodium

butyrate was omitted in all experiments. Media were changed every 2 days, and after 3 to 5 days, cell cultures were photographed with a Nikon ELWD 0.3 phase contrast microscope on Kodak T-Max film (100 X magnification).

Quantification of cord lenth and branching.

TAC-2 cells were suspended at $5x10^3$ or $1x10^4$ cells/ml incollagen gels (500 µl) cast into 16-mm wells of 4-well plates (Nunc, Kampstrup, Rosklide, Denmark) and incubated in 500 µl complete medium in the presence or the absence of 10ng/ml HGF. After 7 days, the cultures were fixed with 2.5% glutaraldehyde in 0.1M cacodylate buffer, and at least 3 randomly selected fields (measuring 2.2 mm x 3.4 mm) per experimental condition in each of 3 separate experiments were photographed with a Nikon Diaphot TMD inverted photomicroscope. The total length of the cords present in each individual colony was measured with a Qmet 500 image analyzer (Leyca Cambridge Ltd., Cambridge, UK). Cord length was considered as "0" in: a) colonies with a spheroidal shape, and b) slightly elongated structures in which the length to diameter ratio was less than 2. Quantification of branching was performed by counting all branch points in each colony. Values of cord length and branching obtained from the largest colonies are an underestimate, since in these colonies a considerable proportion of cords were out of focus and therefore could not be measured. Values were expressed either as mean cord length and number of branch points per photographic field [170] or as mean cord length and number of branch points per individual colony [166]. The mean values for each experimental condition were compared to controls using the Student's unpaired T-test.

Immunoblot analysis.

HA-tagged Notch4(int-3), Notch4(int-3) deletion mutants and Wnt-1 proteins from lysates of TAC-2 cell populations were analyzed by immunoblotting. To maximize protein expression, TAC-2 cells were treated with 2mM sodium butyrate for 16 hours prior to lysis. Cells were washed twice with cold PBS and then removed from dishes in 1.5 ml PBS using a rubber policeman. Cells were pelleted by centrifugation at 2,000x g at 4°C for 5 min. and lysed in 90 µl TENT buffer (20 mM Tris, pH 8.0, 2 mM EDTA, 150 mM NaCl, 1% Triton-X100) containing 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin, 2 mg/ml leupeptin, 1 mg/ml pepstatin, at 4°C for 30 min. Lysates were clarified by centrifugation at 10,000xg at 4°C for 10 min., and protein contents were determined using the BioRad Protein determination kit. Lysates containing 40 µg protein were electrophoresed in 10% SDS-polyacrylamide gels. Proteins were transferred from gels onto nitrocellulose by electroblotting, and then blocked overnight at 4°C in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 0.2% Tween-20) containing 1% bovine serum albumin (fraction V). Blots were then incubated in anti-HA monoclonal antibody (12CA5) diluted 1:100 in TBST at room temperature. After four hours, the blot was washed three times for 5 min. each in TBST. Blots were exposed to a 1:16,000 dilution of HRP-conjugated sheep anti-mouse IgG. Blots were washed as above and then incubated 1-2 min. in enhanced chemiluminescence reagents (Amersham Inc, IL) and exposed to X-ray film (Fujifilm, Fuji Photo Film Co., LTD., Tokyo).

Figure legends for chapter VI.

Figure 1

Immunoblot analysis on TAC-2 cell lysates using anti-HA antibodies. TAC-2 cells programmed to express either LacZ, Wnt-1 or int-3 were grown in the presence or absence of sodium butyrate. Wnt-1 and int-3 proteins of appropriate molecular weight can be detected in the respective TAC-2 cell lines, and the level of protein expression can be increased by sodium butyrate treatment.

Figure 2

TAC-2 cell ductal morphogenesis assay. TAC-2 cells programmed to express LacZ (A,B,C), Wnt-1HA (D,E,F), or int-3HA (G,H,I) were grown in collagen gels either in the absence of exogenous growth factor (A,D,G), in the presence of HGF (B,E,H), or TGF–β2 (C,F,I). HGF and TGF–β2 induce branching morphogenesis of TAC-2 LacZ cells (compare A to B or C). Wnt-1 expression in TAC-2 cells induces modest branching morphogenesis of TAC-2 cells in the absence of either HGF or TGF–β2 (compare D to A), and robust branching is observed when TAC-2 Wnt-1 cells are grown in the presence of either HGF or TGF–β2 (compare E to B, and to C). TAC-2 cells programmed to express int-3 fail to undergo branching morphogenesis when grown in the presence of either HGF or TGF–β2 (compare G to H or I, H to B, and I to C).

Figure 3
Wnt-1 and HGF have cooperative effects on branching morphogenesis of TAC-2 cells, while expression of int-3 inhibits HGF-induced branching morphogenesis. TAC-2 LacZ, TAC-2 Wnt-1 and TAC-2 int-3 cells were suspended in collagen gels at $5x10^3$ cells/ml (A and B) or $1x10^4$ cells/ml (C and D) and incubated with either control medium or 10ng/ml HGF for 7 days. In each of 3 separate experiments, at least three randomly selected fields per condition were photographed. The total additive length of all cords in each field (A), the number of cord branch points per field (B), the total additive length of all cords in each individual colony (C), and the number of cord branch points per colony (D) was determined as described in Materials and Methods. Values are mean ± s.e.m.; n=3. Values for HGF are significantly ($P<0.001$) different when compared to controls (except for TAC-2 int-3 cells) and values are significantly different ($P<0.001$) when TAC-2 LacZ and TAC-2 Wnt-1 cell lines are compared. Similar results were obtained by evaluating cord length and branching per individual TAC-2 LacZ andTAC-2 Wnt-1 colony (data not shown).

Figure 4
Differential behavior of TAC-2 LacZ cells and TAC-2 Wnt-1 cells in hydrocortisone-supplemented cultures. Cells were suspended in collagen gels at $5x10^3$ cells/ml and incubated for 10 days with 1 μg/ml hydrocortisone and 50 ng/ml cholera toxin. Under these conditions, TAC-2 LacZ cells form thick-walled spheroidal cysts enclosing a widely patent lumen (A,C,E), as previously shown for untransfected cells. In marked contrast, TAC-2 Wnt-1 cells form branched structures consisting of either short tubules (B), cords containing small mutifocal lumina (D) or apparently solid cords (F). The three-dimensional structures illustrated in A,C,E and B,D,F are representative of the vast majority of colonies formed by TAC-2 LacZ and TAC-2 Wnt-1 cells, respectively. Magnification = 180x.

Figure 5
Schematic representation of int-3 deletion mutants (A) and immunoblot analysis on TAC-2 cell lysates using anti-HA antibodies (B, C). TAC-2 cells programmed to express either DNT, DCDC, DCT, and DNTΔCT were grown in the presence or absence of sodium butyrate. The int-3 deletion proteins are epitope tagged and deletion proteins of appropriate molecular weight can be detected in the respective TAC-2 cell lines, and the level of protein expression can be increased by sodium butyrate treatment. Panel C, immunoblot analysis on lysates of TAC-2 LacZ and TAC-2 DNTΔCT cells, as in panel B, demonstrating the presence of the DNTΔCT deletion protein.

Figure 6
TAC-2 cell ductal morphogenesis assay with int-3 mutants. TAC-2 cells programmed to express ΔNT (A,B), ΔCDC (C,D), ΔCT (E,F), and ΔNTΔCT (G,H) were grown in collagen gels either in the absence of exogenous growth factor (A,C,E,G), or in the presence of HGF (B,D,F,H). HGF induces branching morphogenesis of TAC-2 ΔNT cells (B), TAC-2 ΔCDC cells (D) and TAC-2 ΔNTΔCT cells (H) TAC-2 ΔCT cells fail to undergo branching morphogenesis when grown in the presence of either HGF (F).

Figure 7
TAC-2 cell ductal morphogenesis assay. TAC-2 cells programmed to express LacZ/ctr (A,B,C), LacZ/Wnt-1 (D,E,F), int-3/ctr (G,H,I), or int-3/Wnt-1 (J,K,L) were grown in collagen gels either in the absence of exogenous growth factor (A,D,G,J), in the presence of HGF (20 ng/ml)(B,E,H,K), or in the presence of TGF–β2 (50 pg/ml)(C,F,I,L). HGF and TGF–β2 induce branching morphogenesis of TAC-2 LacZ/ctr cells (compare A to B or C). Wnt-1 expression in TAC-2 cells induces modest branching morphogenesis of TAC-2 LacZ/Wnt-1 cells in the absence of either HGF or TGF–β2 (D), and robust branching is observed when TAC-2 LacZ/Wnt-1 cells are grown in the presence of either HGF (E) or TGF–β2 (F). TAC-2 cells programmed to express int-3/ctr fail to undergo branching morphogenesis when grown in the presence of either HGF (H) or TGF–β2 (I). TAC-2 cells programmed to express both int-3 and Wnt-1 undergo branching morphogenesis in the absence of exogenous growth factor (J), and form a robust branching network when grown in the presence of HGF (K) or TGF–β2 (L) in a similar manner when compared to TAC-2 cells programmed to express Wnt-1 solely (D,E,F).

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Figure 1

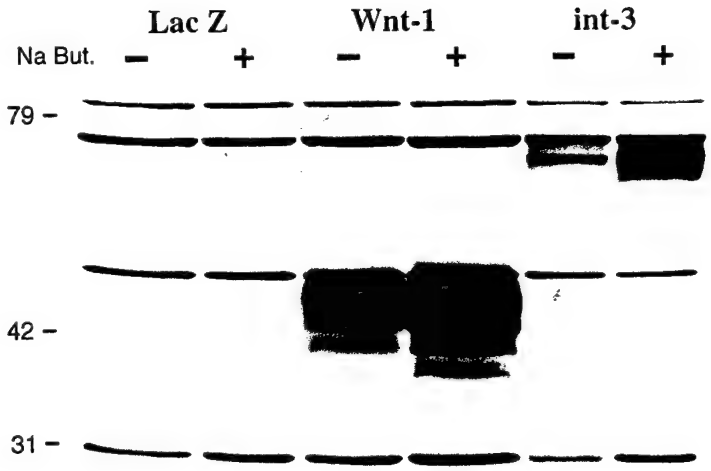

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Figure 2

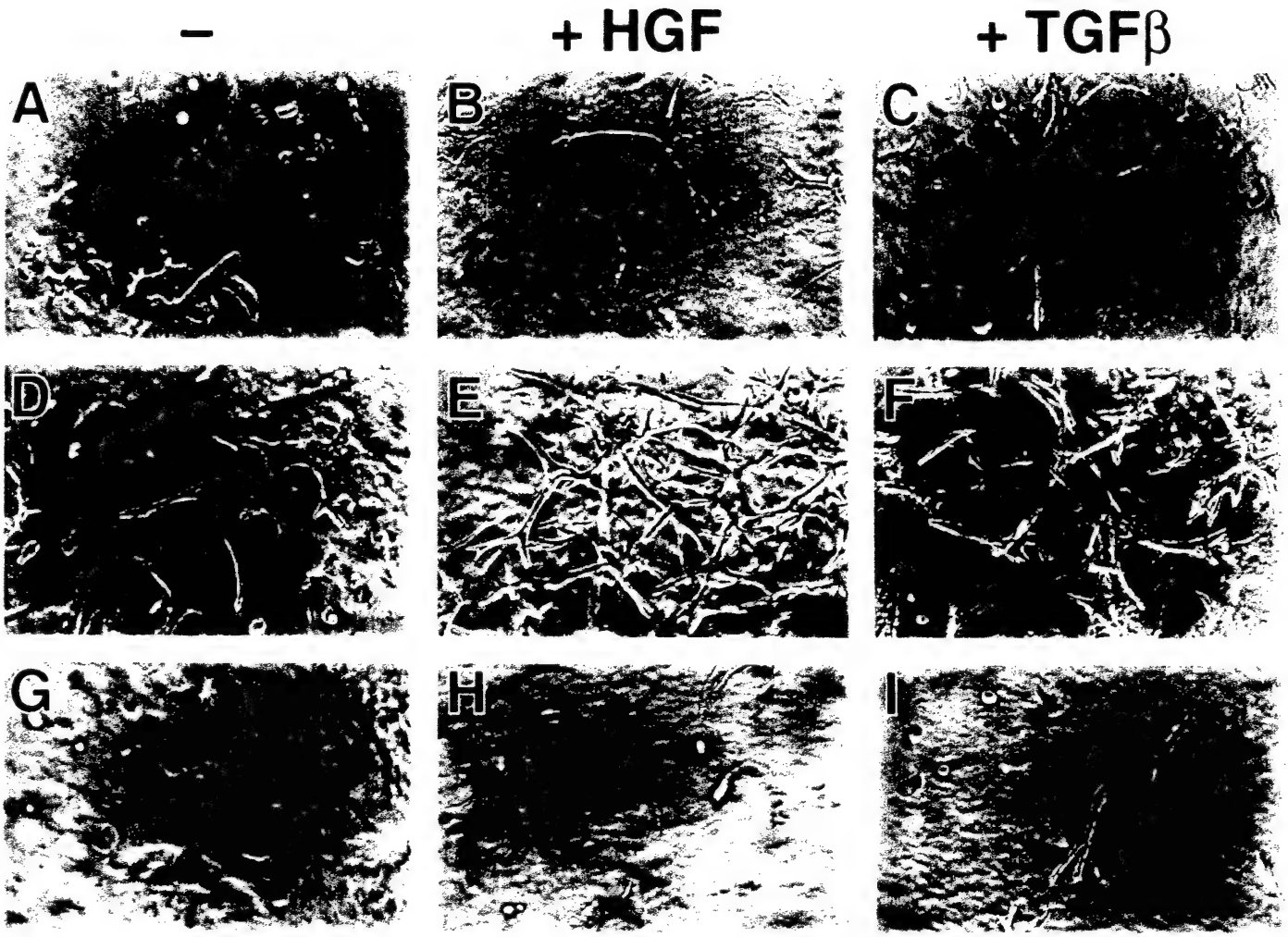

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Figure 3

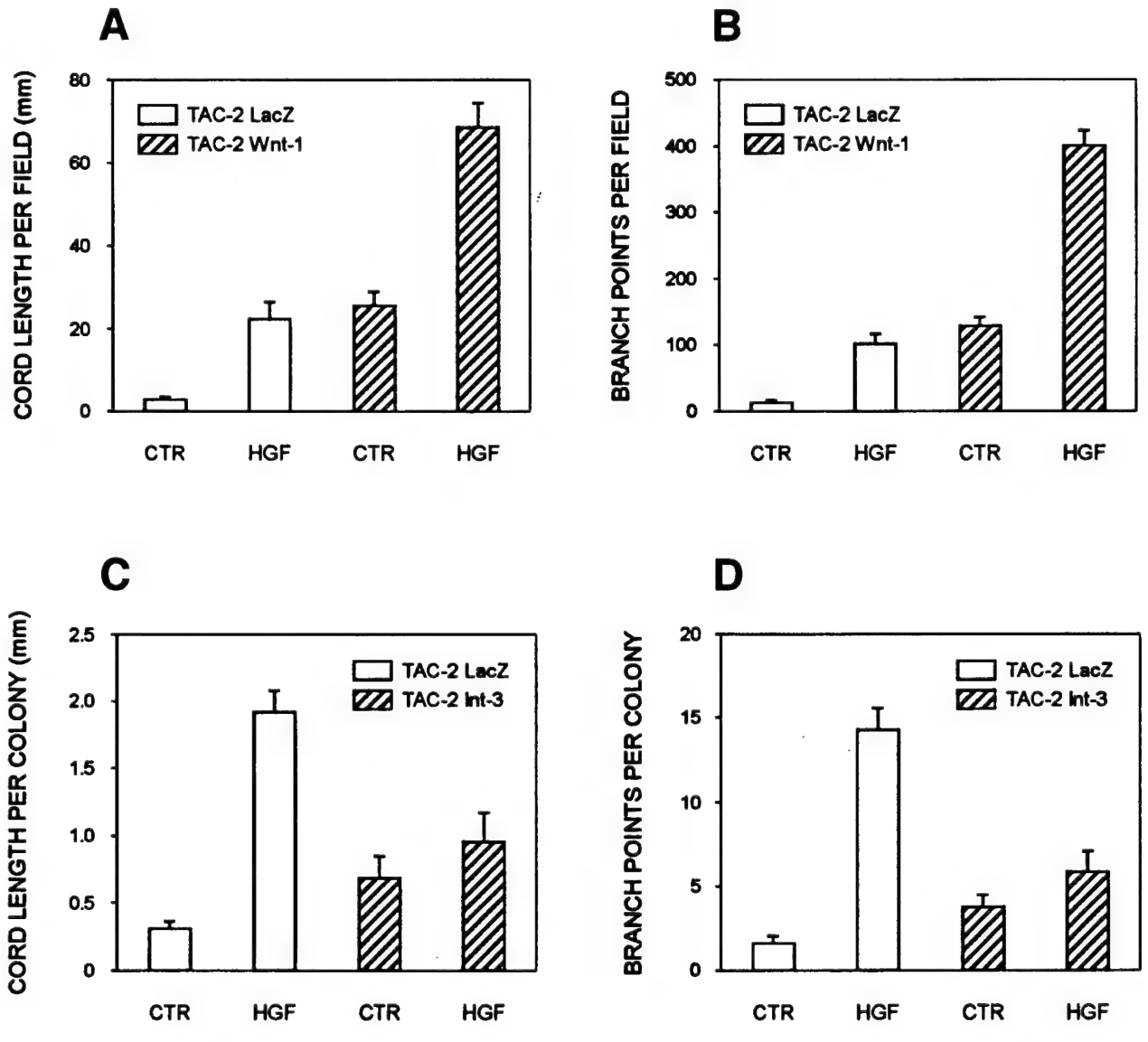

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Figure 4

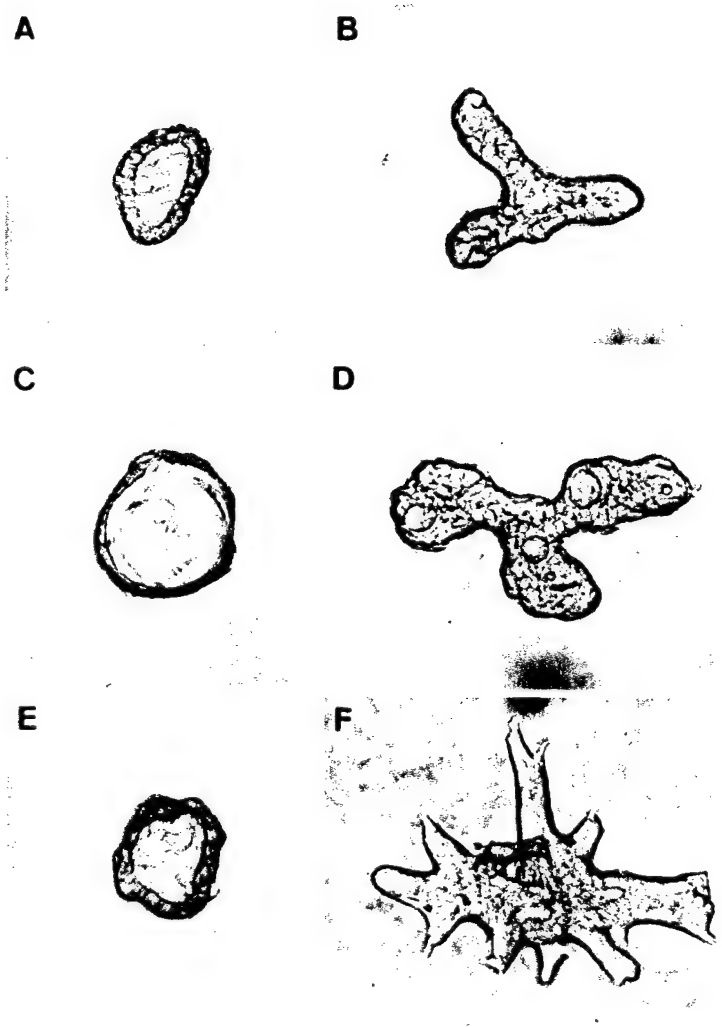

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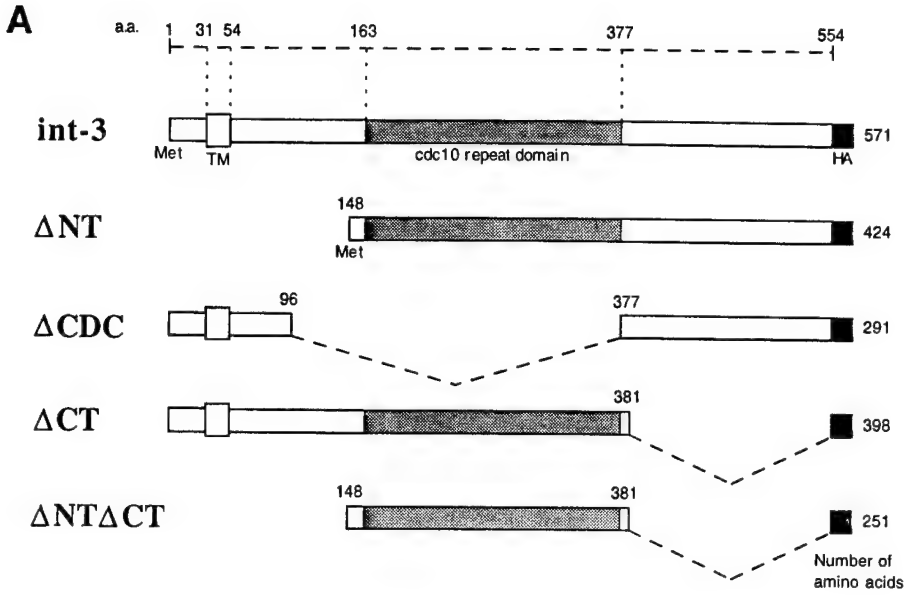
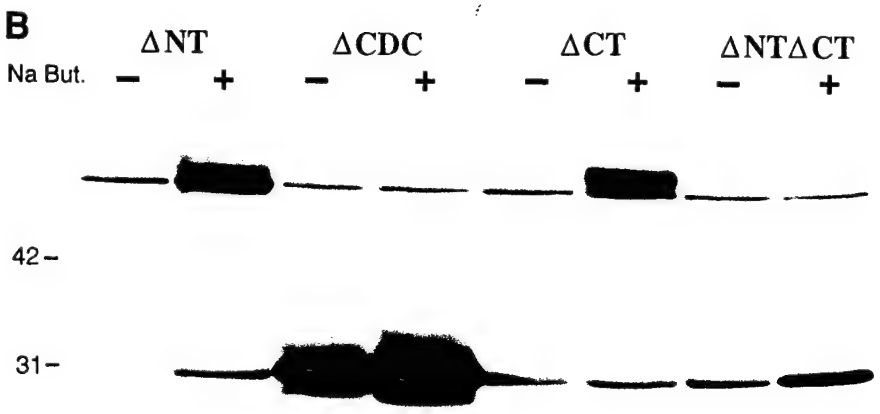
Figure 5





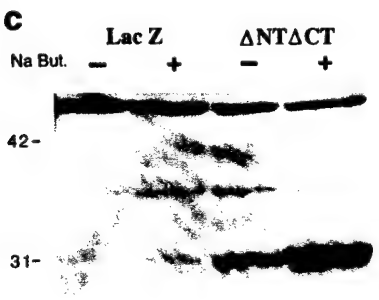


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Figure 6

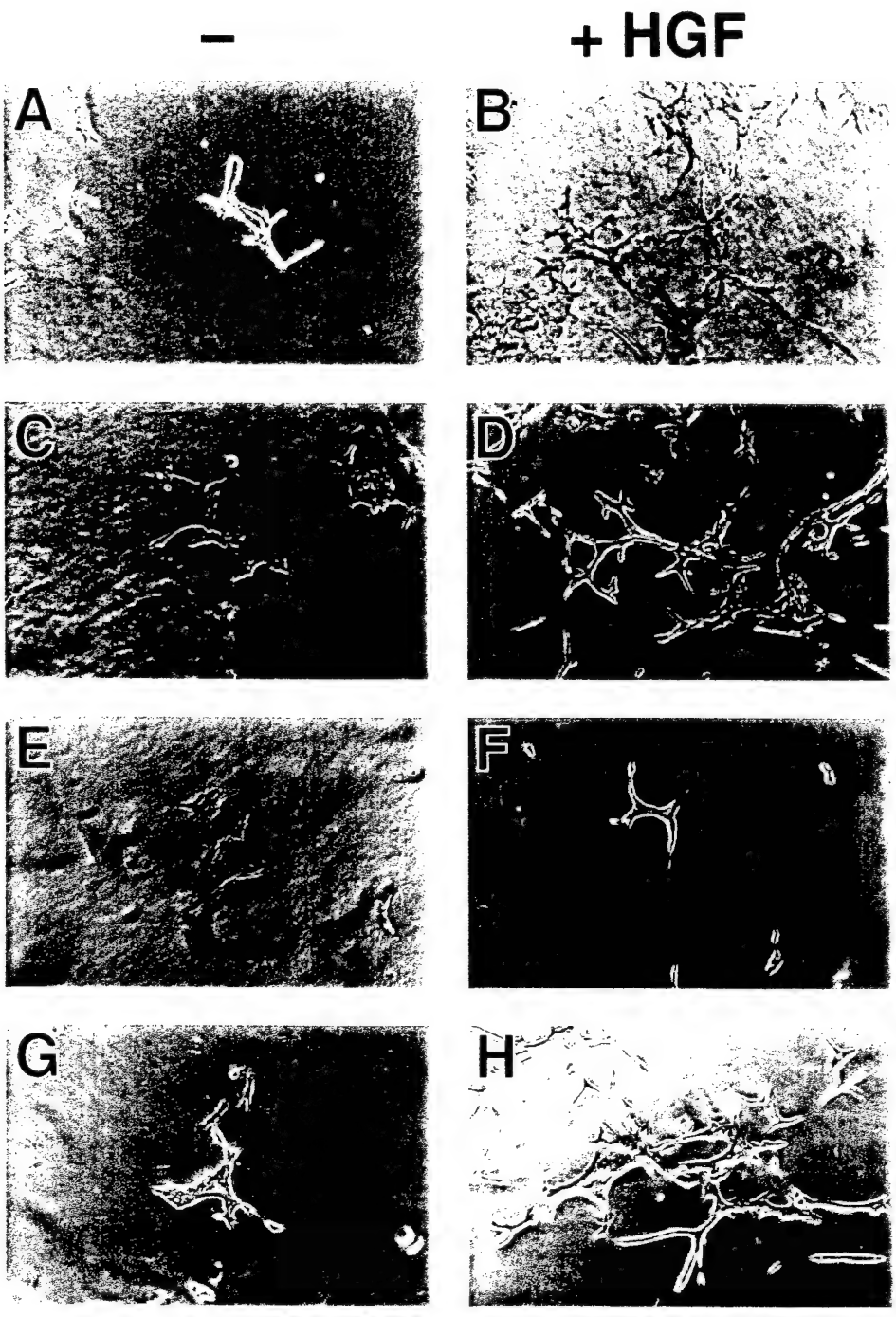

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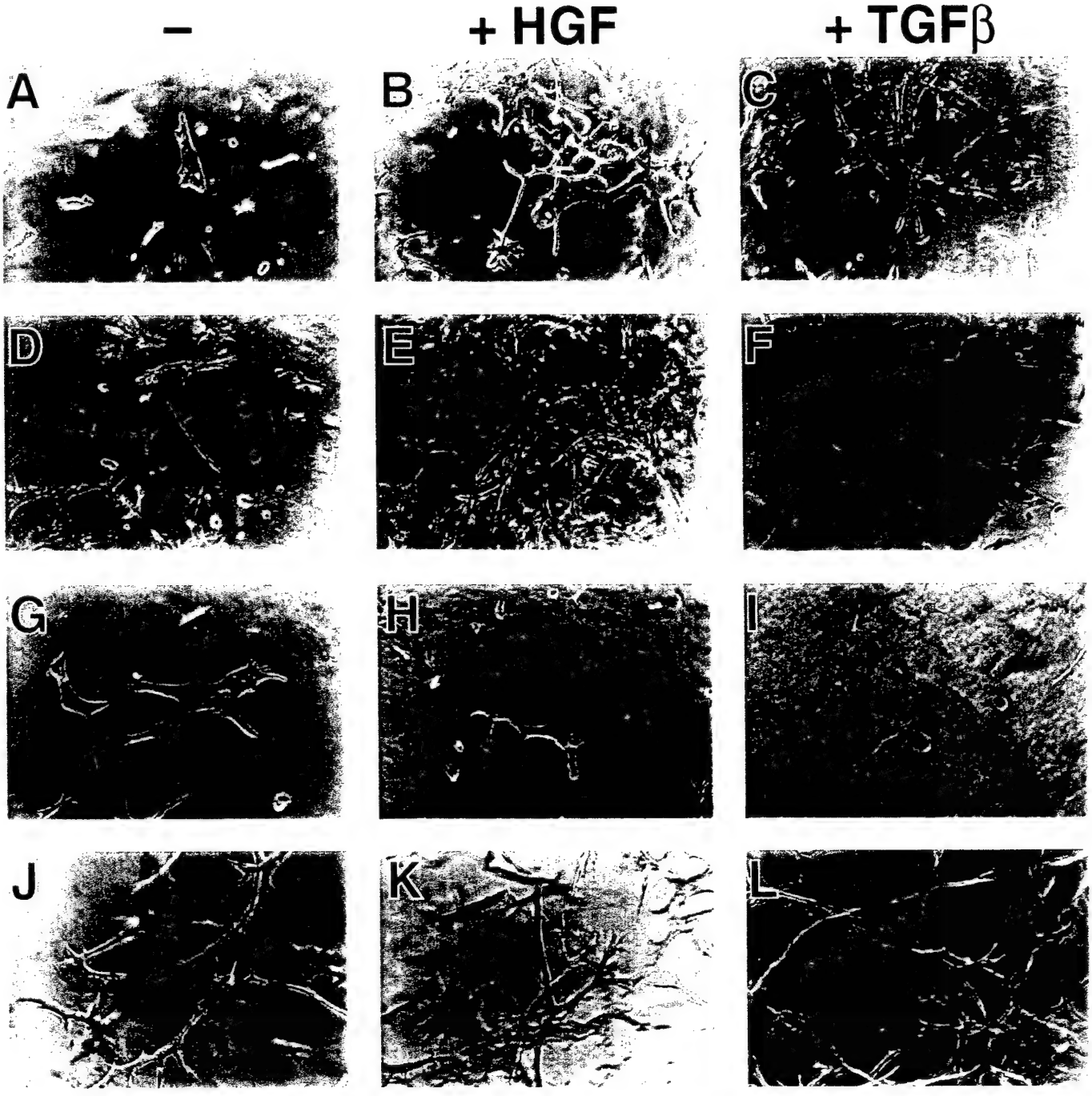
Figure 7



CONCLUSIONS

The data presented in this annual report represents results of experiments outlined in specific aim 1-5 of the research proposal. We feel we have completed all of the major objectives in the first three aims. The former specific aims 4-5 are now focused on Wnt signal transduction and on the interaction between the frizzled-like protein, frzb, and Wnt proteins. Significant progress has been made in this area. We have documented a physical interaction between frzb and Wnt-1 and have begun to map the domains required for Wnt-frzb association. In addition, we have begun to define the events that lead to regulation of β–catenin levels and activity.

In conclusion, we have segregated Wnt proteins into functional classes based upon their ability to transform mammary epithelial cells. This segregation may represent classes of Wnt proteins that interact with distinct Wnt-cell surface receptors. This may represent the first type of evidence that their may be distinct Wnt-cell surface receptors. Alternatively, one class may be involved in mitogenic stimulus and are thus are transforming, whereas the other class may be involved in differentiation of the mammary epithelium. Two interesting aspects of the segregation come out of this analysis. First, it appears that those Wnt genes either not normally expressed in the mammary gland (Wnt-1, Wnt-3, Wnt-3A, and Wnt-7B) or expressed at very low levels in the mammary gland (Wnt-2) are the most transforming. Whereas, those Wnt genes that are well expressed in the mammary gland (Wnt-4, Wnt-5A, Wnt-5B, Wnt-6) do not exhibit transformation activity. Second, when one compares the activity of the transforming genes to those reported to be overexpressed in mammary tumors [70] only one Wnt gene that is not transforming is overexpressed in this study. Wnt-2, Wnt-3, and Wnt-7B were all found to be transforming in our hands and have been found to be overexpressed in several mammary tumors; however, Wnt-4 never displayed transforming activity in our experiments but was found to be overexpressed in mammary tumors. The transforming potential correlates with the ability of Wnt family proteins to increase levels of cytosolic β–catenin. Thus, Transformation may depend on the ability of Wnt proteins to alter β–catenin levels. These findings indicate that upregulation of cytosolic β–catenin is dependent on expression of the transforming class of Wnt proteins, providing the first evidence that increased cytosolic β–catenin correlates with cellular transformation.

To identify regions within the 370 amino acid Wnt-1 protein required for these functions we tested eleven chimeric genes that contained variable amounts of Wnt-1 and Wnt-5a sequence. Transformation and β–catenin regulation in C57MG cells is controlled by amino acids that lie within 186 residues of the amino terminus of Wnt-1. Small substitutions between residues 186 and 292 reduced Wnt-1 activity. Replacement of the carboxy terminal 79 amino acids of Wnt-1 by Wnt-5a did not affect function. These results were supported by transient expression asssays in 293 cells wherein β–catenin accumulated in the cytoplasm in response to ectopic Wnt-1 expression. In 293 cells, a larger region of the amino-terminus of Wnt-1 was found to be required for β–catenin regulation. Nonfunctional chimeras that contained at least 99 amino terminal Wnt-1 residues inhibited Wnt-1 stimulation of 293 cells. One of these chimeras inhibited both Wnt-1 and Wnt-3 activity suggesting that Wnt-1 and Wnt-3 interact with a common signaling component.

We demonstrate that Frzb-1 blocks Wnt-1 induced cytosolic accumulation of ß-catenin, a key component of the Wnt signaling pathway, in human embryonic kidney cells. Structure/function analysis reveals that complete removal or partial deletions of the frizzled domain of Frzb-1 abolishes its inhibition of Wnt-1 activity. Deletion of the C-terminal domain of Frzb decreases its blocking efficiency.

We show that Wnt-1 induces a growth response in a cultured mammalian cell line, Rat-1 fibroblasts. Wnt-1 induces serum-independent cellular proliferation of Rat-1 fibroblasts and changes in morphology. Rat-1 cells stably expressing Wnt-1 (Rat-1/Wnt-1) show a constitutive up-regulation of cytosolic β–catenin, while membrane-associated β–catenin remain unaffected. Induction of cytosolic β–catenin in Rat-1/Wnt-1 cells is correlated with activation of a Tcf-responsive transcriptional element. We thus provide evidence that Wnt-1 induces Tcf/Lef transcriptional activation in a mammalian system. Expression of a mutant β–catenin (β–CatS37A) in Rat-1 cells does not result in a proliferative response or a detectable change in the cytosolic β–catenin protein level. However, β–CatS37A expression in Rat-1

cells results in strong Tcf/Lef transcriptional activation, comparable to that seen in Wnt-1 expressing cells. These results suggest that Wnt-1 induction of cytosolic β–catenin may have functions in addition to Tcf/Lef transcriptional activation.

Elongation and branching of epithelial ducts is a crucial event during the development of the mammary gland. Branching morphogenesis of the mouse mammary epithelial TAC-2 cell line was used as an assay to examine the role of Wnt, HGF, TGF–β and the Notch receptors in branching morphogenesis. Wnt-1 was found to induce the elongation and branching of epithelial tubules, like HGF and TGF–β2, and to strongly cooperate with either HGF or TGF–β2 in this activity. Wnt-1 displayed morphogenetic activity in TAC-2 cells as it induced branching even under conditions that normally promote cyst formation. The Notch4(int-3) mammary oncoprotein, an activated form of the Notch4 receptor, inhibited the branching morphogenesis normally induced by HGF and TGF–β2. The minimal domain within the Notch4(int-3) protein required to inhibit morphogenesis consists of the CBF-1 interaction domain and the cdc10 repeat domain. Co-expression of Wnt-1 and Notch4(int-3) demonstrates that Wnt-1 can overcome the Notch-mediated inhibition of ductal morphogenesis. These data suggest that Wnt and Notch signaling may play opposite roles in mammary gland development, a finding consistent with the convergence of the wingless and Notch signaling pathways found in Drosophila.

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PERSONNEL FOR THIS EFFORT

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